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Development of a Microfluidic Paper-Based Device for the Detection and Quantification of Aluminium in Urine: Implications for Alzheimer's Disease Prevention and Diagnosis

By
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Thesis presented to the *Escola Superior de Biotecnologia, Universidade Católica Portuguesa*, in partial fulfilment of the requirements for the Master of Science degree in Biomedical Engineering.

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Resumo

Esta tese apresenta uma avaliação do desenvolvimento e aplicação de um *microfluidic paper-based analytical device (μPAD)* para a deteção quantitativa de alumínio na urina, com consideráveis implicações para a pesquisa e diagnóstico da doença de Alzheimer (DA). O estudo baseia-se na hipótese da implicação do alumínio na patogénese da DA, que indica uma relação causal potencial entre o acumular de alumínio nos tecidos neurais e o início dos processos neurodegenerativos.

A metodologia de investigação inclui uma abordagem diversificada, começando com uma avaliação crítica da urina como uma matriz biológica não invasiva para a quantificação de alumínio. É realizada uma análise estruturada das abordagens analíticas atuais, incluindo espectrometria de absorção atómica (AAS) e espectrometria de emissão ótica por plasma acoplado indutivamente (ICP-OES), para estabelecer um modelo de desempenho analítico e identificar áreas para potenciais melhorias.

O núcleo da pesquisa foca-se no design, fabricação e otimização de um novo μPAD. Este dispositivo utiliza os princípios da ação capilar e da deteção colorimétrica para executar uma quantificação rápida e sensível de alumínio. O desempenho do μPAD é rigorosamente avaliado através da comparação com métodos analíticos estabelecidos, com ênfase particular na sensibilidade, especificidade e reprodutibilidade em matrizes urinárias complexas.
A chave da inovação deste trabalho centra-se na integração de um sistema de reagente cromogénico cuidadosamente selecionado dentro do substrato de papel, permitindo a quantificação visual e instrumental do alumínio. O estudo esclarece os mecanismos químicos essenciais à reação colorimétrica e explora estratégias para aumentar a intensidade e estabilidade do sinal. Análises estatísticas são empregues para confirmar o desempenho analítico do μPAD, incluindo o limite de deteção (LOD) de 0,12 mg/L, limite de quantificação (LOQ) de 0,40 mg/L, *linear range* e variabilidade inter- e intra-ensaio. A robustez do dispositivo é ainda avaliada através de estudos de recuperação e análise de amostras reais de urina de um grupo de dadores.

Este dispositivo contribui para o diagnóstico da DA ao fornecer uma ferramenta económica, portátil e fácil de usar para a deteção de alumínio na urina. O μPAD desenvolvido mostra potencial tanto para aplicações clínicas quanto epidemiológicas, podendo facilitar a deteção precoce de níveis elevados de alumínio e a monitorização da progressão da DA. Além disso, este trabalho estabelece as bases para futuras investigações sobre a utilidade do alumínio na urina como biomarcador para a avaliação do risco da DA e monitorização terapêutica.

Palavras-chave:

microfluidic paper-based analytical device (μPAD); Deteção de alumínio; Análise de urina; Doença de Alzheimer (DA); Processos neurodegenerativos; Matriz biológica não invasiva; Ação capilar; Deteção colorimétrica; Sistema de reagente cromogénico; Biomarcador.

Abstract

This thesis reports a complete assessment of the development and application of a microfluidic paper-based analytical device (μPAD) for the quantitative detection of aluminium in urine, with considerable implications for Alzheimer's disease (AD) research and diagnostics.

The core of the research focuses on the design, fabrication, and optimisation of a novel μPAD. This device utilizes the principles of capillary action and colorimetric detection to reach rapid and sensitive aluminium quantification. The μPAD's performance is rigorously assessed against established analytical methods, with particular emphasis on sensitivity, specificity, and reproducibility in complex urinary matrices. A key innovation of this work is centred on the integration of a carefully selected chromogenic reagent system within the paper substrate, enabling visual and instrumental quantification of aluminium. The study clarifies the chemical mechanisms essential the colorimetric reaction and explores strategies to enhance signal intensity and stability. Statistical analyses are employed to confirm the μPAD's analytical performance, including limit of detection (LOD) of 0.12 mg/L, limit of quantification (LOQ) of 0.40 mg/L, linear range, and inter- and intra-assay variability. The device's robustness is further evaluated through recovery studies and analysis of real urine samples from a cohort of subjects.

This research contributes to the field of AD diagnostics by providing a cost-effective, portable, and user-friendly tool for aluminium detection in urine. The developed μPAD shows potential for both clinical and epidemiological applications, potentially facilitating early detection of elevated aluminium levels and monitoring of AD progression. Furthermore, this work lays the groundwork for future investigations into the utility of urinary aluminium as a biomarker for AD risk assessment and therapeutic monitoring.

Key words: Microfluidic paper-based analytical device (μPAD); Aluminum detection; Urine analysis; Alzheimer's disease (AD); Neurodegenerative processes; Non-invasive biological matrix; Capillary action; Colorimetric detection; Chromogenic reagent system; Biomarker;

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1. Introduction

1.1. Urine Samples

The use of biological samples for monitoring health conditions has traditionally focused on blood due to its rich information content. However, there is a growing interest in non-invasive biological samples, such as urine, saliva, and sweat, which offer several advantages over invasive methods. Non-invasive sampling techniques significantly reduce patient discomfort and anxiety, making them more acceptable to a wider population. Additionally, these methods facilitate easier and more frequent sample collection, which is essential for monitoring chronic conditions or conducting large-scale epidemiological studies. The ability to collect samples at home or in non-clinical settings enhances accessibility and convenience for patients, particularly in resource-limited environments.

Urine is a particularly valuable biological fluid for clinical applications. Composed primarily of water (approximately 95%), urine also contains urea (around 2%), creatinine (0.1%), uric acid (0.03%), and various ions and molecules such as chloride, sodium, potassium, sulphate, ammonium, and phosphate[1]. This composition not only reflects the metabolic state of the body but also provides insights into renal function and systemic health. The advantages of using urine include its ease of collection, stability in composition compared to other biofluids like plasma, and the ability to obtain large volumes without discomfort to the patient. Furthermore, urine proteomics has emerged as a significant field for biomarker discovery in various diseases, including renal disorders such as diabetic nephropathy and acute kidney injury[2,3].
Despite these advantages, there are some disadvantages associated with urine analysis. The concentration of certain biomarkers may be lower in urine than in blood, potentially complicating the detection of some conditions. Additionally, urine composition can vary significantly due to factors such as hydration status, diet, and time of day, which may affect the reliability of test results[4, 5] .

Clinically, urine is used extensively for diagnostic purposes through urinalysis - a simple yet powerful tool that examines the visual, chemical, and microscopic properties of urine. Urinalysis plays a crucial role in diagnosing conditions such as urinary tract infections (UTIs), kidney diseases, diabetes mellitus, and even systemic diseases[4,5]. It allows for the detection of abnormalities such as proteinuria (excess protein), haematuria (blood in urine), and glucosuria (glucose in urine), which can indicate underlying health issues[5].
Moreover, advancements in analytical techniques have expanded the scope of urine testing beyond traditional urinalysis to include innovative methods such as Fourier Transform Infrared (FTIR) spectroscopy for early disease detection[1].
In summary, while blood remains a primary sample type for health monitoring due to its comprehensive information content, non-invasive alternatives like urine present significant advantages in terms of patient comfort and accessibility. The diverse applications of urine analysis in clinical settings underscore its importance as a diagnostic tool and its potential for enhancing personalized medicine approaches

1.2. Aluminium

Aluminium is a lightweight metal that belongs to the 13th group of the periodic table[6]. Human exposure to aluminium is relatively high, once it is the 3rd most abundant element in the earth crust[7]. Aluminium is commonly encountered in various forms, including air, water, food, soil, and consumer products[40].

All individuals have trace amounts of this metal in their bodies, which can be measured in blood, bone tissue, faeces, and urine[6, 8]. Assessing aluminium levels in blood and urine can help determine if a person has been exposed to higher-than-normal concentrations of aluminium[6, 9].
The primary route of aluminium exposure for the general population is through the consumption of food. However, smaller amounts of exposure may also occur through ingestion of aluminium in drinking water and inhalation of ambient air[10].

1.2.1. Alzheimer's Disease and Aluminum Hypothesis

The aluminium hypothesis proposes a potential link between aluminium exposure and the development of Alzheimer's disease (AD), a neurodegenerative disorder defined by cognitive decline and memory loss. Although the exact mechanisms remain unclear, aluminium is considered a neurotoxin that may aggravate oxidative stress and inflammatory responses in the brain, leading neurodegeneration[11].

Research has shown that aluminium can inhibit various biological processes, involving neurotransmitter synthesis and gene expression, which are crucial for neuronal health[12]. Historical studies have indicated that aluminium accumulation in the brains of AD patients has a correlation with the presence of neurofibrillary tangles and amyloid plaques, major pathological characteristics of the disease[13].

As an illustration, Klatzo et al. demonstrated that the intracerebral administration of aluminium in animal models led to neurofibrillary degeneration analogous to that observed in human AD cases[14]. Although there remains debate about the robustness of this association, accumulating evidence suggests that long-term exposure to aluminium is perhaps a significant environmental factor influencing the pathogenesis of AD[15]. Epidemiological studies have reported higher incidences of AD in populations with elevated aluminium levels in drinking water, further supporting this hypothesis[16]. However, it is important to note that while some studies have found a correlation between aluminium exposure and AD, others have failed to establish a definitive causal relationship, leading to continued controversy in the scientific community regarding aluminium's role in AD[17].

1.2.2. Biomarkers in urine for Alzheimer's disease

Recent investigation has identified potential biomarkers in urine that could aid in the early diagnosis of Alzheimer's disease (AD). These biomarkers include various proteins and metabolites that reflect pathological processes occurring in the brain, particularly the accumulation of amyloid-beta (Aβ) and phosphorylated tau (p-Tau)[18]. Elevated levels of these neurodegeneration-related proteins have been detected in urine samples from AD patients, suggesting their potential as non-invasive diagnostic tools[19]. The non-invasive nature of urine collection presents a significant advantage over traditional diagnostic methods, making it an attractive option for large-scale screening and monitoring of disease progression[20]. For instance, studies have demonstrated that urinary Aβ and p-Tau levels correlate with cognitive decline and the presence of amyloid plaques in the brain, which are hallmarks of Alzheimer's pathology[20].

Furthermore, ongoing studies aim to validate these urinary biomarkers to enhance their reliability and clinical applicability in diagnosing Alzheimer's disease[21]. The development of microfluidic paper-based analytical devices (μPADs) can facilitate the detection of these biomarkers, allowing for rapid and cost-effective analysis without the need for complex laboratory equipment. This technological advancement holds promise for improving early diagnosis and monitoring of AD, ultimately contributing to better patient management and treatment strategies.

As research progresses, the integration of urinary biomarkers into clinical practice could revolutionise how Alzheimer's disease is diagnosed and monitored, providing a more accessible means for healthcare providers to assess neurodegenerative conditions in patients[22]. Additionally, understanding the dynamics between these biomarkers may offer deeper insights into the pathophysiology of Alzheimer's disease and its progression[23].

1.2.3. Methods for aluminum analysis in biological samples

Analytical techniques play a crucial role in the identification and quantification of various analytes in a wide range of samples, mainly in fields such as environmental monitoring, clinical diagnostics, and materials science. Part of these techniques, atomic absorption spectrometry (AAS) and inductively coupled plasma optical emission spectrometry (ICP-OES) are widely utilised for their precision and reliability. AAS is distinguished for its ability to measure specific wavelengths of electromagnetic radiation absorbed by elements, allowing for high-precision elemental evaluation[24]. In contrast, ICP-OES excels in multi-element detection and is particularly effective for analysing samples with higher concentrations of dissolved solids[25]. Recognising the strengths and limitations of these practices is key for selecting the appropriate technique for individualized analytical necessities.

Atomic absorption spectrometry is a technique used for the identification and quantification of elements in liquid or solid samples by applying specific wavelengths of electromagnetic radiation emitted from a light source[24]. By utilizing the specific wavelengths absorbed by different atoms, AAS provides precise detection and measurement of elements in a sample[24]. This adaptability makes AAS suitable for both trace and high-concentration analyses with robust precision[26].

Despite its advantages, atomic absorption spectrophotometry is limited to analyse one element at a time, making it less effective for multi-element analysis. Another aspect to consider is the relatively high expense associated with AAS equipment[24, 26]. The initial purchase represents a significant investment and another obstacle, particularly for laboratories with restricted budgets; and the ongoing costs for maintenance and regular calibration add to the overall expenditure [26].

On the other hand, for the detection of aluminium in urine, ICP-OES is a reliable method, especially when samples consistently contain concentrations exceeding 10 ppb[25]. When analysing samples with elevated levels of total dissolved solids (TDS) or suspended particles, although ICP-MS is capable of measuring these, ICP-OES offers a simpler and more routine approach[25]. Moreover, for laboratories processing large volumes of samples exceeding 1,200 per day ICP-OES is advantageous due to its rapid analysis speed, requiring less than one minute to measure the concentration of up to 74 elements in a single sample[25]. Although the ICP-OES method offers robust analytical capabilities, the equipment and operating costs are relatively high[27]. Samples commonly must be converted into a solution prior to analysis, as direct injection of solid samples can lead to reduced precision and accuracy[27]. In like manner, the advantages of ICP-OES might not be prominent for certain elements[27].

1.3. Microfluidic paper-based device (µPAD)

Microfluidic paper-based analytical devices (µPADs) have emerged as a promising platform for point-of-care diagnostics, offering unique advantages over traditional microfluidic systems[46, 47]. These devices leverage the inherent properties of paper substrates to create cost-effective, portable, and user-friendly diagnostic tools that align with the World Health Organization's ASSURED criteria (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable to end-users)[48]. µPADs utilise cellulose-based materials as substrates, enabling capillary-driven fluid transport without the need for external pumps or power sources[46, 47]. This passive flow mechanism, coupled with the ability to store reagents directly on the paper, allows for simplified assay protocols and reduced user intervention[47]. Their design and functionality allow for point-of-care analysis, enabling tests to be conducted without the need for complex laboratory equipment, thus facilitating their use in diverse environments, including remote areas with limited access to advanced technology. µPADs require only small sample volumes, which is particularly advantageous for biological samples where the availability of material may be limited. Additionally, they utilize a reduced quantity of reagents, making them more eco-friendly compared to

conventional methods. Their portability further enhances their utility, allowing for application in various settings worldwide. Furthermore, µPADs are not only cost-effective but also remarkably easy to use, making them accessible to a broader range of users.

Microfluidics, involving the manipulation of fluids within micro-scale channels, has emerged as an influential field poised to impact chemical synthesis, biological analysis, optics, and information technology[28]. With microfluidics still in its developmental phase, there are several critical challenges to address, including selecting and focusing on practical applications and creating pathways to commercialisation[28]. Resolving these issues will demand imaginative approaches, as this technology holds promise for broad applications in healthcare, environmental protection, and diagnostics[28].

Among microfluidic innovations, microfluidic paper-based analytical devices (µPADs) and micro total analysis systems are particularly promising. These devices enable the efficient analysis of complex biochemical samples, including macromolecules, proteins, nucleic acids, toxins, cells, and pathogens[17]. Within a single analytical cycle, µPADs can perform various fluidic processes, such as transport, sorting, mixing, and separation[29]. As a result, they are well-suited for diverse applications, including neurotransmitter detection, cancer diagnostics, cell and tissue culture, drug discovery, and the identification of microorganisms[30] . The versatility of µPADs is further enhanced by their compatibility with various detection methods, including colorimetric, electrochemical, and fluorescence-based techniques[47]. The fabrication of µPADs has been significantly simplified through the development of accessible patterning methods[47]. These include photolithography, wax printing, and more recently, laser printing techniques, which allow for rapid prototyping and potential mass production of devices[48, 49].
The ability to create complex microfluidic networks on paper substrates has enabled the design of multiplexed assays capable of detecting multiple analytes simultaneously[47]. In the field of diagnostics, µPADs have demonstrated remarkable potential for a wide range of applications, including the detection of infectious diseases, biomarkers for non-communicable diseases, and environmental contaminants[46, 47]. Their utility in resource-limited settings is particularly noteworthy, as they provide a means for conducting sophisticated analytical tests without the need for expensive laboratory equipment or highly trained personnel[47].

Given their potential for rapid, cost-effective biochemical analysis, µPADs and lab-on-chip devices are now a focal point in research, with implications for medical sample screening and forensic diagnostics[28].

Global health and environmental protection remain critical priorities; however, high healthcare costs and a lack of diagnostic tools pose barriers in both developed and developing regions[30]. In low-resource settings, infectious diseases that would be treatable in more advanced healthcare systems often result in severe outcomes due to limited access to diagnostics and trained personnel[29, 30]. This creates an urgent need for affordable, easy-to-use point-of-care (POC) diagnostic methods to support effective diagnosis and patient monitoring[28]. In environmental monitoring, a similar gap exists; lab-on-chip technology offers the potential to enhance global health through accessible POC testing and to promote environmental protection by enabling analytical assessment of environmental samples[28]. As genetic research and molecular biology advance, high-throughput single-

cell analysis will further drive insights into cell biology, providing a basis for more comprehensive diagnostic tools and targeted healthcare strategies[28, 17]

Recent advancements in µPAD technology have focused on improving sensitivity, specificity, and quantification capabilities[47]. Integration with smartphone-based readout systems has further enhanced the analytical power of these devices, allowing for rapid data acquisition, analysis, and transmission[47]. This convergence of paper-based microfluidics with digital technologies is paving the way for connected diagnostics and telemedicine applications[47]. As the field of µPADs continues to evolve, challenges such as standardisation of fabrication processes, long-term reagent stability, and integration with sample preparation steps are being actively addressed by researchers [47].
The ongoing development of novel materials, detection strategies, and device designs promises to expand the capabilities of µPADs, potentially revolutionising point-of-care diagnostics and enabling widespread access to affordable healthcare solutions[47]
The next figure shows two examples of the developed µPADs.

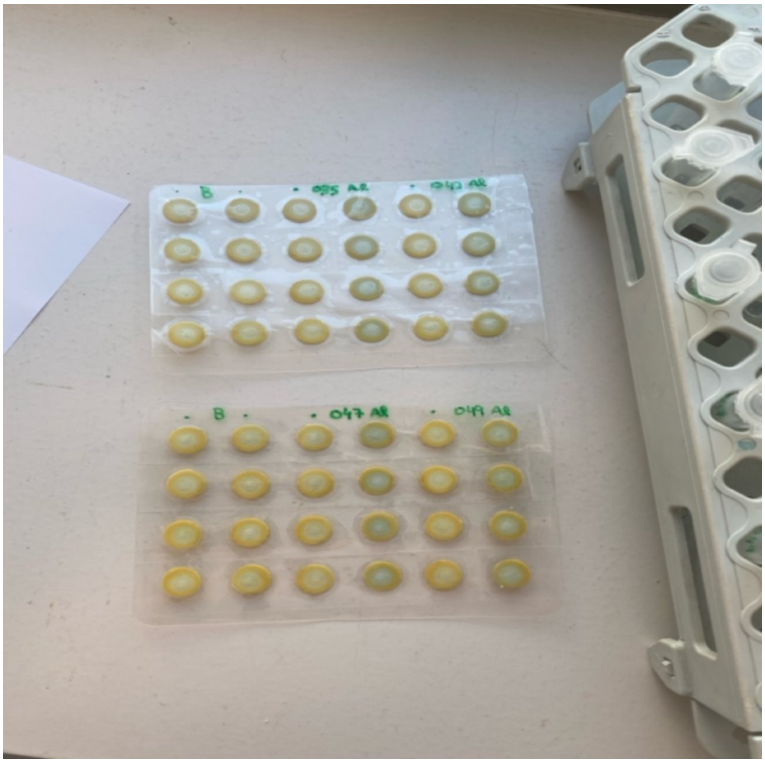

Figure 1- aluminium quantification µPADs

1.3.1. Methods employed for μPADs assembly

The fabrication of μPADs can be achieved through various techniques, including wax printing, cutting, and inkjet printing. These methods allow for the creation of intricate fluidic pathways on paper substrates. Additionally, μPADs can be classified into 2D (lateral) and 3D (vertical) designs. The vertical flow approach is particularly notable as it enables more complex channel designs and can integrate multiple functionalities within a single device. This versatility in design and construction contributes to the growing application of μPADs across various fields, including biomedical diagnostics and environmental monitoring.
In summary, the development and utilization of μPADs represent a significant advancement in analytical technology, offering practical solutions that align with sustainable practices while maintaining efficiency and effectiveness in diverse applications.

1.3.2. Detection Methods in Microfluidic Paper-Based Analytical Devices

Microfluidic paper-based analytical devices have become prominent tools in analytical chemistry due to their versatility and ease of use. Various detection methods can be integrated into μPADs, each offering unique advantages for specific applications. Below is a detailed overview of several methods employed in μPADs, including electrochemical, fluorometric, chemiluminescent, surface plasmon resonance (SPR), photothermal, lateral flow assay (LFA)-based methods, and enzyme-linked reactions.
Electrochemical methods in μPADs involve the integration of electrodes into the device to measure electrical signals generated during chemical reactions[17]. These methods are particularly suitable for quantifying ions, metabolites, or biomolecules such as DNA and proteins. Common techniques include amperometry and voltammetry. Amperometry measures the current produced by the oxidation or reduction of an analyte at a specific electrode potential, while voltammetry involves varying the potential and measuring the resulting current to provide information about the analyte's concentration[34]. The use of electrochemical sensors in μPADs allows for rapid analysis with high sensitivity, making them valuable tools in clinical diagnostics and environmental monitoring.

Fluorometric methods utilise fluorescent dyes or markers to detect specific analytes within μPADs[34]. This approach requires a UV or visible light source and a detector to measure emitted fluorescence. The high sensitivity of fluorometric techniques allows for the detection of low-abundance biomolecules, making them particularly useful for DNA and protein analysis. In this context, fluorophores can be conjugated to antibodies or nucleic acid probes that specifically bind to target molecules, resulting in a measurable fluorescent signal upon excitation[33]. The ability to perform multiplexed assays using different fluorescent markers further enhances the utility of this method in complex biological samples.

Chemiluminescent methods generate light as a result of chemical reactions occurring within the μPAD[32]. These techniques are highly sensitive and capable of detecting low concentrations of analytes without requiring external light sources. The inherent luminescence produced during these reactions can be quantified using photodetectors or

imaging systems. This method is particularly advantageous for applications requiring high sensitivity, such as immunoassays for disease biomarkers[35]. The simplicity of these assays, combined with their rapid response times, positions chemiluminescent μPADs as powerful tools in clinical diagnostics.
Surface plasmon resonance (SPR) methods exploit the plasmonic properties of metallic nanoparticles integrated into μPADs to detect biomolecular interactions or changes in refractive index[36]. When light hits a metal surface at a specific angle, it can excite surface plasmons - coherent oscillations of free electrons at the metal-dielectric interface. Changes in refractive index due to binding events on the surface lead to shifts in the resonance angle, which can be precisely measured. This technique is particularly useful for real-time monitoring of interactions between biomolecules, making it invaluable in research settings focused on drug development and biomolecular characterisation.

Photothermal methods involve heat generation from light absorption by nanoparticles integrated into μPADs[37]. When illuminated with a laser or other light source, these nanoparticles absorb energy and convert it into heat, leading to local temperature changes that can be detected optically or thermally. This technique is beneficial for sensitive detection applications when combined with spectroscopy, allowing for enhanced signal-to-noise ratios in measurements. Photothermal methods are increasingly being explored for applications such as pathogen detection and environmental monitoring due to their high sensitivity and rapid response times[37].

Lateral flow assay (LFA) - based methods integrate microfluidic paper-based analytical devices (μPADs) with principles derived from traditional lateral flow assays. These devices are widely employed in rapid diagnostics, including pregnancy tests and COVID-19 antigen tests. The design typically consists of a sample pad where liquid samples are introduced, followed by a conjugate pad containing labelled antibodies that bind to target analytes. As the sample flows along the strip via capillary action, it interacts with immobilised capture antibodies on a test line, generating a visible signal if the target is present[17]. The simplicity and speed of LFA-based μPADs make them ideal for point-of-care testing scenarios, allowing for quick results without the need for complex laboratory equipment[37]. Recent advancements have further enhanced the sensitivity and specificity of these assays, making them suitable for a broader range of applications beyond infectious disease detection, including food safety and environmental monitoring[33].

Enzyme-linked reactions utilise enzymes immobilised on paper substrates within μPADs to catalyse reactions that produce measurable signals. For example, glucose oxidase can be used for glucose detection; when glucose is present, it undergoes enzymatic oxidation producing hydrogen peroxide, which can then react with chromogenic substrates to yield a colour change detectable via imaging techniques[38]. This method allows for highly specific and sensitive detection while maintaining simplicity and ease of use. The incorporation of enzyme-linked reactions into μPADs has enabled the development of rapid diagnostic tests that require minimal sample volumes and provide results in a short time frame[39]. The versatility of this approach allows it to be applied in various fields, including clinical diagnostics and food safety testing.

1.4. Significance of Aluminum Detection in Urine

The detection of aluminium in urine has emerged as a crucial aspect of toxicological and occupational health research[40, 41]. This analytical determination will serve as a vital tool for assessing chronic exposure to aluminium, particularly in cases of occupational or environmental exposure[9]. The significance of aluminium detection in urine lies in its ability to provide a reliable indicator of aluminium concentrations in the body, especially when measured one or two days after exposure[9].

Whilst normal exposure to small amounts of aluminium is generally considered harmless, excessive exposure or accumulation in the body can lead to detrimental health effects[41]. The kidneys typically eliminate absorbed aluminium through urine, but individuals with diminished kidney function or those exposed to high levels of aluminium may be at risk of toxicity[40, 41].

The 24-hour urine test for aluminium has become a standard method for assessing an individual's exposure levels and evaluating potential risks associated with aluminium toxicity[41]. This test is particularly valuable in monitoring chronic exposure, as it offers higher sensitivity compared to other biological specimens[9].

The urinary aluminium concentration can provide insights into the body burden of aluminium and serve as an early indicator of potential health complications[41, 9].

Analytical techniques for aluminium detection in urine have evolved significantly, with inductively coupled plasma mass spectrometry (ICP-MS) emerging as the gold-standard determination method[9]. This technique offers superior quantification limits, selectivity, and robustness compared to other methods[9].

The development and implementation of reliable methods for aluminium determination in clinical laboratories are crucial for accurate assessment and interpretation of results[9]. Understanding the significance of aluminium detection in urine is essential for various fields, including occupational health, environmental toxicology, and clinical medicine[40, 41, 9]. It enables the early detection of aluminium toxicity, facilitates the monitoring of exposure in high-risk populations, and aids in the development of preventive strategies [41]. Furthermore, it contributes to our understanding of the potential long-term health effects of aluminium exposure and informs public health policies[37, 38].

As research in this area continues to advance, the significance of aluminium detection in urine is likely to grow, potentially leading to improved diagnostic tools, more effective interventions, and enhanced occupational safety measures[38, 9].

This introduction sets the stage for a comprehensive exploration of the methods, applications, and implications of aluminium detection in urine, highlighting its importance in safeguarding human health in an increasingly aluminium-rich environment.

1.5. Challenges in Aluminium Detection

The detection of aluminium in biological samples presents several significant challenges that researchers and clinicians must address to ensure accurate and reliable results[42, 41]. These challenges span various aspects of the analytical process, from sample collection to result interpretation. One of the primary challenges in aluminium detection is the risk of sample contamination[43].

Aluminium is ubiquitous in the environment, and even minute amounts of external contamination can significantly skew test results. This necessitates rigorous protocols for sample collection and handling, including the use of specialised, non-metallic containers and acid-washed equipment[43].

The choice of analytical technique poses another challenge. Whilst inductively coupled plasma mass spectrometry (ICP-MS) has emerged as the gold standard for aluminium detection, its high sensitivity can be both an advantage and a complication[43]. The extreme sensitivity of ICP-MS requires meticulous attention to potential sources of interference and contamination throughout the analytical process. Interpretation of results presents a significant challenge, particularly in the context of provoked urine testing[44].

The lack of standardised reference ranges for provoked urine samples in healthy subjects makes it difficult to draw meaningful conclusions from these tests[44].

Furthermore, the unpredictable nature of aluminium excretion following chelator administration complicates the interpretation of results and their clinical significance[44]. The variability in testing protocols and chelating agents used in provoked urine testing further compounds the challenges in aluminium detection[44]. The absence of a standardised, validated challenge test makes it difficult to compare results across different studies or clinical settings[44]. Another critical challenge lies in distinguishing between current exposure and total body burden of aluminium[45]. Whilst unprovoked urine samples can provide insight into recent exposure, they may not accurately reflect the total body burden of aluminium accumulated over time[45].

This limitation necessitates careful consideration when interpreting test results and their implications for patient health. The potential for adverse reactions to chelating agents used in provoked urine testing presents both ethical and practical challenges[44]. These risks must be carefully weighed against the questionable diagnostic value of such tests, particularly given the lack of evidence supporting their use in diagnosing metal toxicity[44].

In brief, the challenges in aluminium detection are multifaceted, encompassing technical, interpretative, and ethical considerations. Addressing these challenges requires a

concerted effort from researchers and clinicians to develop standardised protocols, establish appropriate reference ranges, and critically evaluate the clinical utility of various testing methods.

1.6. Objective of the Study

The objective of this study was to develop a microfluidic paper-based analytical device (µPAD) for the quantification of aluminium (Al^{3+}) in urine, utilising the colour reagent Chrome Azurol S (CAS). This device aims to provide a low-cost, rapid and accessible method for detecting trace metals in biological samples.

The µPAD is designed to assess whether a patient's urine contains potentially harmful concentrations of aluminium ions (Al^{3+}), facilitating health evaluations without requiring technical expertise from the user. This contrasts with traditional methods such as atomic absorption spectroscopy or inductively coupled plasma (ICP) aluminium analysis, which are more complex.

Colourimetric methods are widely employed in µPADs due to their ability to provide both qualitative and quantitative results based on visual changes in colour intensity. Upon introduction of the sample containing Al^{3+} ions into the µPAD, the CAS reagent reacts with these ions to produce a distinct colour change that can be easily observed. The intensity of this colour change is directly proportional to the concentration of Al^{3+} present in the urine sample[31]. Challenges associated with colourimetric detection on µPADs include ensuring uniform colour distribution across detection zones to avoid discrepancies in readings due to effects such as coffee ring formation or non-uniform shapes of coloured areas[32, 33].

Despite the limited reaction space inherent to the device, its sensitivity is designed to detect only concentrations harmful to health, effectively addressing the limitation of the detection range. Also, this method is advantageous due to its simplicity, minimal reagent requirements, and the capability to provide quantifiable results without the need for extensive laboratory infrastructure. This selective approach enhances the device's efficiency, ensuring that the response is relevant solely to cases of potential health risk, thereby minimizing false positives at subclinical levels. Furthermore, it reinforces the utility of the µPAD as a practical screening tool with significant implications for the monitoring of populations exposed to environmental sources of aluminium, without necessitating detailed quantification of low concentrations that lack immediate clinical relevance.

This feature enables the µPAD to remain an efficient and viable solution for aluminium detection in biological samples, particularly for the screening of cases with a potential higher risk associated with Alzheimer's disease.

2. Materials and Methods

2.1. Reagents and solutions

The solutions were all prepared with analytical grade chemicals and distilled water.

An aluminium stock solution of 0.37mmol/L (10 mg/L) was prepared by diluting the atomic absorption commercial standard of 1000 mg/L.

The reagent was obtained by dissolving 40 mg of CAS, chrome azurol S (Sigma-Aldrich, Germany) in 10 mL of water, obtaining a final concentration of 4g/L.

On the paper tests, de the reagent was constituted by adding 250 µL of CAS, 250 µL of water and 500 µL of buffer solution.

The buffer solution was composed of 13.6 g of sodium acetate anhydrous (Merck, Germany) dissolved in water, combined with 4.0 mL of a 1M acetic acid solution (Merck, Germany). The mixture is then diluted to a final volume of 100 mL. This combination of sodium acetate (a conjugate base) and acetic acid (a weak acid) creates a buffer system capable of maintaining a stable pH, which in this case is adjusted to 6.5[56].

An intermediate aluminium standard solution of 10 mg/L was prepared from the atomic absorption standard of 1000 mg/L in synthetic urine. From this solution, aluminium standard solutions at concentrations ranging from 0.2 mg/L to 2 mg/L were prepared in water.

To prepare the synthetic urine solution it was weighed 0.1 g of lactic acid, 0.4 g of citric acid, 10 g of urea, 0.07 g of uric acid, 0.8 g of creatinine, 0.37 g of bi-hydrated calcium chloride, 5.2 g of sodium chloride, 0.49 g of magnesium sulfate·$7H_2O$, 1.41 g of sodium sulfate·$10H_2O$, 0.95 g of potassium hydrogen phosphate, and 1.2 g of di-potassium hydrogen phosphate. Each component was then dissolved in distilled water and subsequently added to a 1 L volumetric flask. The solution was made up to the 1 L mark with distilled water[56].

2.2. µPAD assembly

The µPAD is composed of two layers of paper, which make up the hydrophilic part of the device.
The two-layer unit (hydrophilic zone) is isolated from the hydrophobic zones using plastic lamination pouches (75 x 110 x 0.125 mm, Q-Connect, Gent, Belgium). One µPAD contains 4 rows with 6 holes in each row, amounting to a total of 24 holes. The opposite side of the pouch does not contain any hole. Each disc has a diameter of 9.5 mm, obtained using a 3/8" paper punch (EK Tools, Lindon, USA).

The next step in assembling the device involved placing 15 µL of colour reagent (CAS) onto all the discs of the lowermost layer (R). The discs with reagent were then placed in an oven at 50°C for 15 minutes to dry.
Finally, to complete the assembly, the discs with and without reagent (R and E, respectively) were stacked, and each pair of discs was placed inside the plastic pouch, aligned with the holes where the samples would be applied (figure 1 (A)). The uppermost disc, without reagent, has the function of reservoir to allow a larger sample volume to be added, and acts as a filtration barrier for sediments present in the urine (E). The hydrophilic and hydrophobic sections of the device are composed by the two-layer disc section and the plastic pouch, respectively.
The hydrophobic zone was established using a laminating process (A3-330C High Quality Laminator), by sealing each paper unit (#24) ensuring a clear separation between the hydrophobic and hydrophilic sections of the device (figure 1(B)).

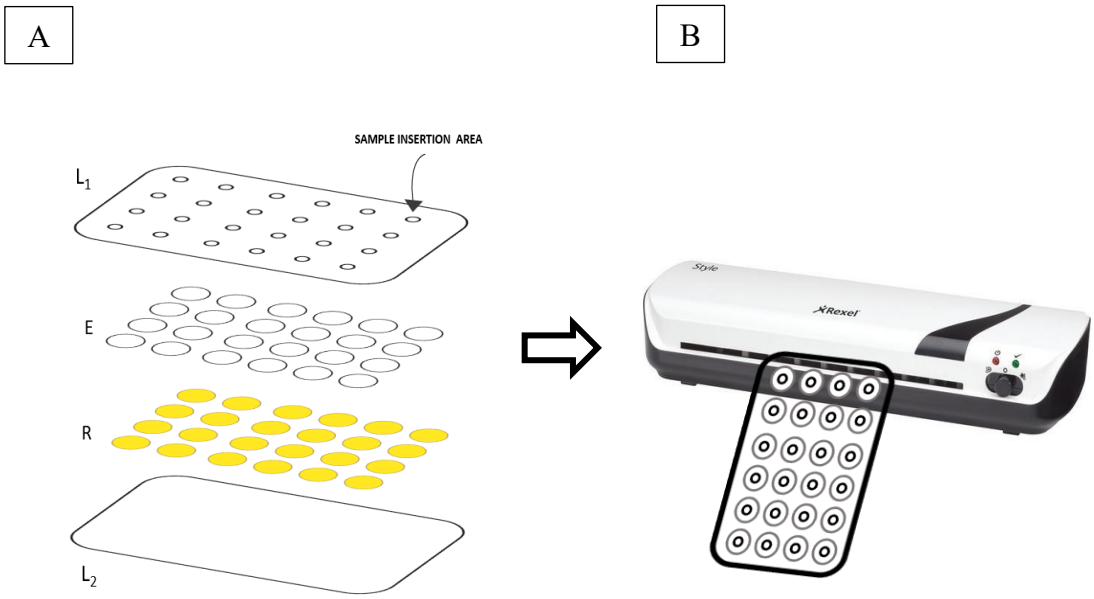

Figure 2 - The µPAD assembly and laminating process; (A) schematic representation of µPAD assembly; (A) Representation of the 4 parts of µPAD 's constitution; (B) Scanning of the device to use ImageJ program.

2.3. Aluminum determination procedure µPAD

A volume of 15 µL of each standard solution or sample was added to each hole. It is important to wait approximately 10 minutes, allowing sufficient time for the colour to develop.
Each column contains standard solutions with increasing concentrations of aluminium. The reaction between CAS and aluminium results in the formation of a blue/green coloured complex, which intensifies as the concentration of aluminium increases (figure

2(B)). When biological samples are used, since the increase of colour depends on aluminium concentration, it could not be noticed.

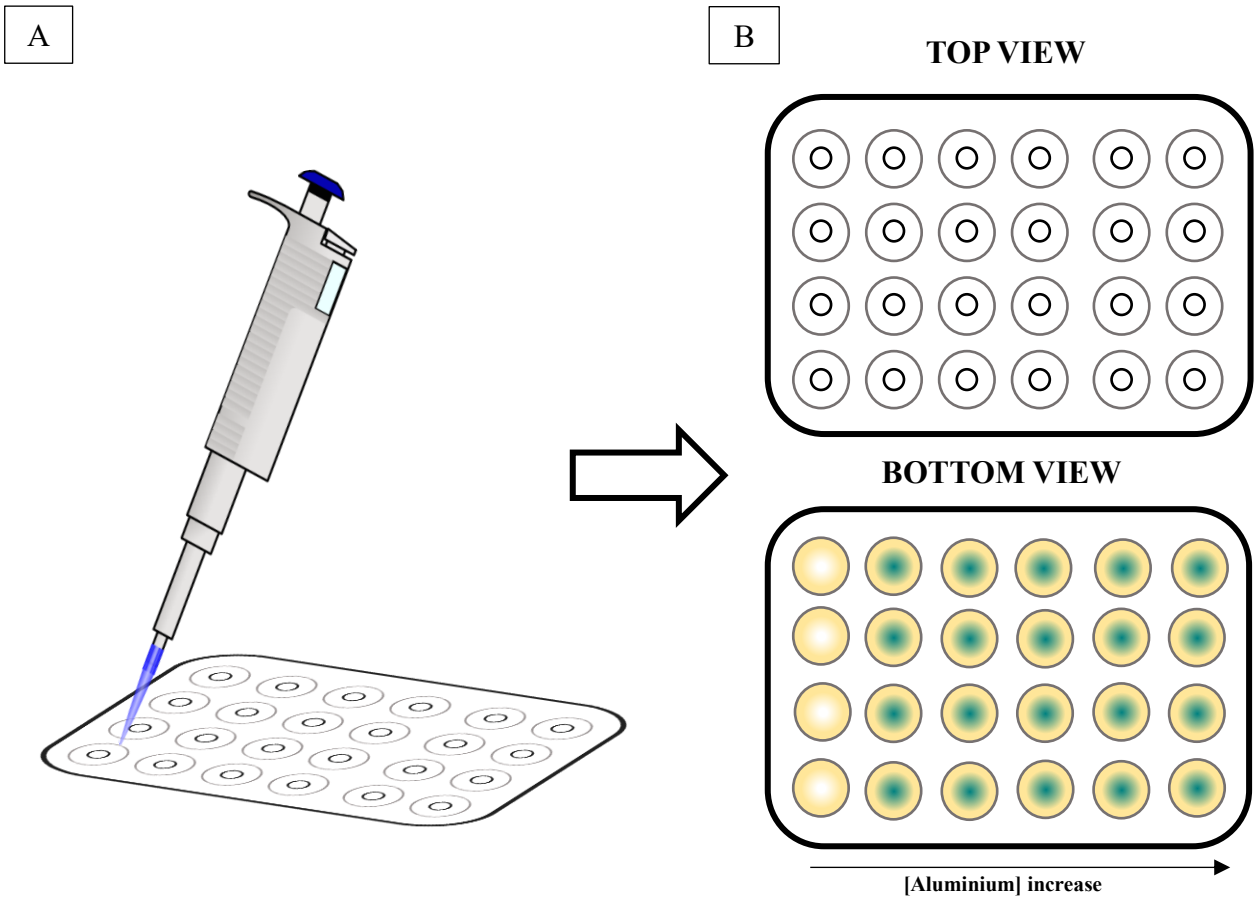

Figure 3 - (A) Sample loading of 15µL; (B) Top and bottom view of a µPAD device; Color intensity and aluminum concentration correlation.

After the 10-minute period adhesive tape was applied over all the holes of the µPAD, so it can protect the operator from touching on the urine samples. The bottom side of the device (L2) was then scanned (Canon LIDE 120) to obtain the detection zone of the µPAD. The bottom side was chosen to be scanned once the adhesive tape constituted a barrier, interfering with the measurements. Also, the fact that the layer E acts like a filter and a reservoir to the reaction, makes the bottom side scanning more precise.
To facilitate accurate quantification of colour intensity on µPADs, image processing techniques are employed. The resulting images are processed using software such as ImageJ, by selecting the RGB Stack filter (red, green, and blue filter). This process involves calculating absorbance for each pixel while eliminating noise caused by the paper substrate [32, 33]. Red filter was chosen for measuring aluminium, in this case, as it is the complementary colour of the product formed (figure 3). The selected area corresponded to the full diameter of the paper disc (figure 3).

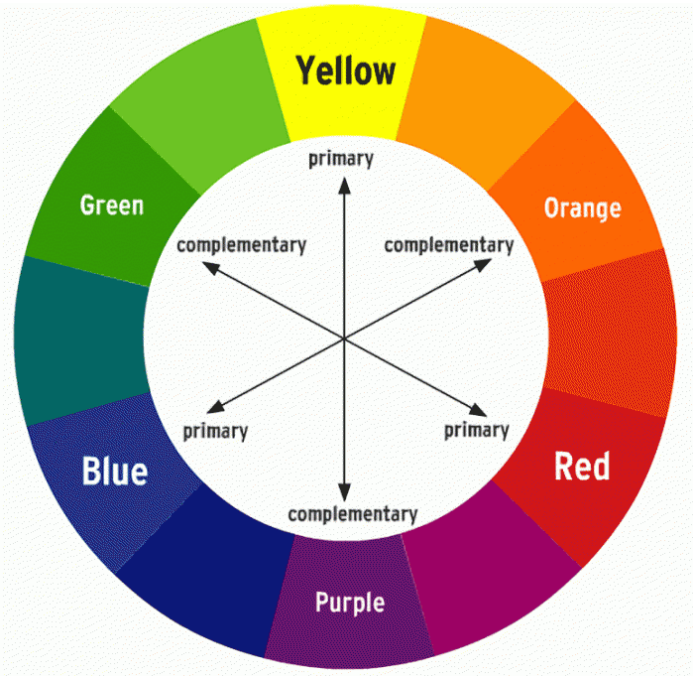

Figure 4 - Primary and complementary colours circle. Used for filter selection.

The intensity values from each disc were then exported to Excel tables. The absorbance values were obtained using the Lambert-Beer equation ($A = log_{10}\frac{I_0}{I}$), where A represents the absorbance value, I_0 denotes the mean colour intensity of the blank solutions, and I refers to the mean colour intensity of the standard or sample solutions[57].

Each intensity value was then converted into concentration using the equation ($A = log_{10}\frac{I_0}{I}$)[57].

By establishing a calibration curve with known concentrations of Al^{3+} solutions prior to testing unknown samples, it is possible to interpolate the concentration based on measured absorbance values from the scanned images[31].

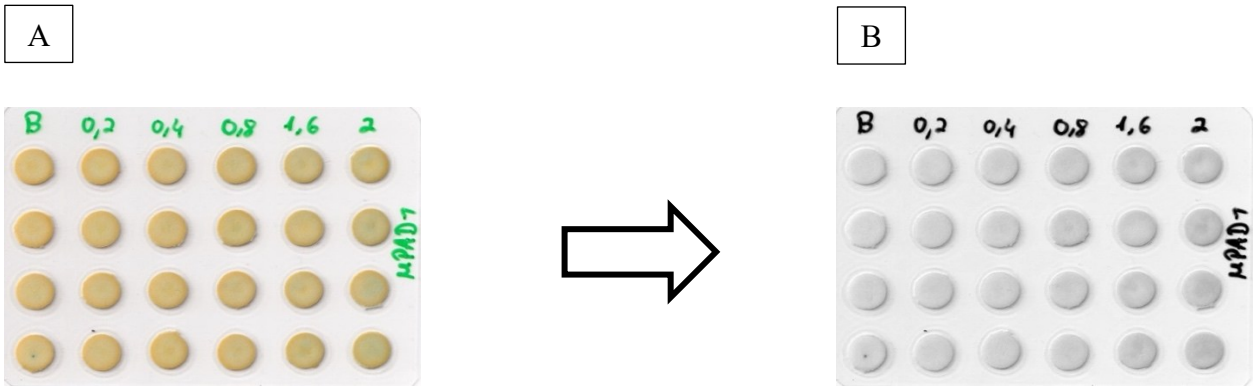

Figure 5 - (A) µPAD after scanning process. (B) Image J filter application, RGB stack for color red. Color intensification with the aluminium concentration increase.

2.4. Batch procedure- preliminary studies

Aluminium standards were prepared in MQW and in synthetic urine using 25 mL volumetric flasks. For each set of tests, six standards with the same range of concentrations were consistently prepared, although the matrix used varied. Each set of six standards included one solution without aluminium (blank). The solutions were transferred into test tubes for measurement using a Hitachi 100-40 UV-Vis spectrophotometer. A Hellma 178.710-QS flow cell, featuring a 10 mm light path and an inner volume of 80 µL, served as the detection system. The wavelength was set to 545 nm for the analysis. Absorbance values were recorded and processed in Excel for the construction of calibration curves. During this testing phase, standards were prepared with various matrices (differing in composition) to determine the optimal conditions for aluminium quantification in urine while minimising potential interferences.

2.5. Sample collection and preparation

The urine samples utilised in this study were collected anonymously without collection of any personal data from the donors, lacking any identification or registered information. These samples were collected from volunteers who provided informed consent. There was no connection to a clinical trial.

Four urine samples were filtered using a 5 mL Braun syringe and a 0.22 µm nylon filter to prevent any precipitate from interfering with subsequent measurements. The filtered samples were then diluted to half their original volume with distilled water.

3. Results and Discussion

To optimize the developed μPAD, the analytical comparison of different features focused on the various slopes (reflecting sensitivity) of calibration curves generated using aluminium standard solutions in the concentration range of 0.2 to 2.0 mg/L. The option with the steepest slope was chosen, as it demonstrated the highest level of sensitivity.

3.1. Reagent Selection - batch studies

The initial study involved comparing different chromogenic reagents for the determination of aluminium, specifically Eriochrome cyanine (ECR)[17] and Chrome Azurol S (CAS)[59, 60]. Calibration curves were established using a batch procedure.
Batch procedure consisted mixing standard solution, buffer solution and reagent for each matrix.

This study aimed to evaluate various reagents to identify the most suitable one for aluminium determination in biological fluids. Initial tests were performed with colour reagents in standard aluminium solutions in both water and synthetic urine. This approach allowed for the determination of the ideal reagent and its concentration.
The initial study involved comparing different chromogenic reagents for the determination of aluminum, specifically Eriochrome Cyanine R (ECR)[17] and Chrome Azurol S (CAS)[59, 60] .
Calibration curves were established using a batch procedure, which involved mixing the standard solution, buffer solution, and reagent for each matrix. The initial phase of testing focused on ECR at a concentration of 1.50 g/L; however, absorbance values could not be obtained due to saturation. To address this issue, a new reagent solution was prepared at 0.15 g/L by diluting the original solution tenfold with water. The calibration curve for the higher reagent concentration exhibited a higher correlation coefficient but demonstrated lower sensitivity, with absorbance values being excessively high for practical use. In contrast, the calibration curves obtained from tests using standard aluminium solutions in water at both concentrations (with pH adjusted using a buffer solution to approximate typical urine pH values) are presented in the following figure. Additionally, this study was conducted using CAS as the reagent at a concentration of 0.41 g/L. Tests performed in water indicated that ECR provided better outcomes than CAS.

A similar study was conducted to evaluate both reagents using aluminium standard solutions prepared in synthetic urine. As with the previous tests, the aluminium standard solutions in water with CAS were buffered to adjust the pH to 6.5.
The results demonstrated significantly greater sensitivity for the reaction with CAS in this matrix. To determine the optimal chromogenic solution, the reaction kinetics were assessed over a 60-minute period through calibration curves, with sensitivity as the primary focus. Although the reaction kinetics of CAS were less extensive than those of ECR, this characteristic proved advantageous. The kinetic evaluation aimed to ascertain whether the extent of the reaction influenced the sensitivity of the calibration curves for

each reagent. The following graph (figure 5) compares the results for each combination of solutions at both 10 minutes and 60 minutes, noting that a waiting period of 10 minutes is required for the reaction to occur, which is designated as time zero). Analysis of the next figure's graph indicates that, over the course of 60 minutes, there were no significant differences in reaction kinetics; however, the sensitivity of the curves remained consistently high, showing similar values for CAS. The subsequent figure presents results using both reagents at time zero (10 minutes) and at 60 minutes. It is evident that CAS exhibits superior sensitivity for aluminium quantification in synthetic urine, and that the reaction kinetics do not positively influence sensitivity, in contrast to ECR in water. These findings support the selection of CAS as the reagent for subsequent studies.

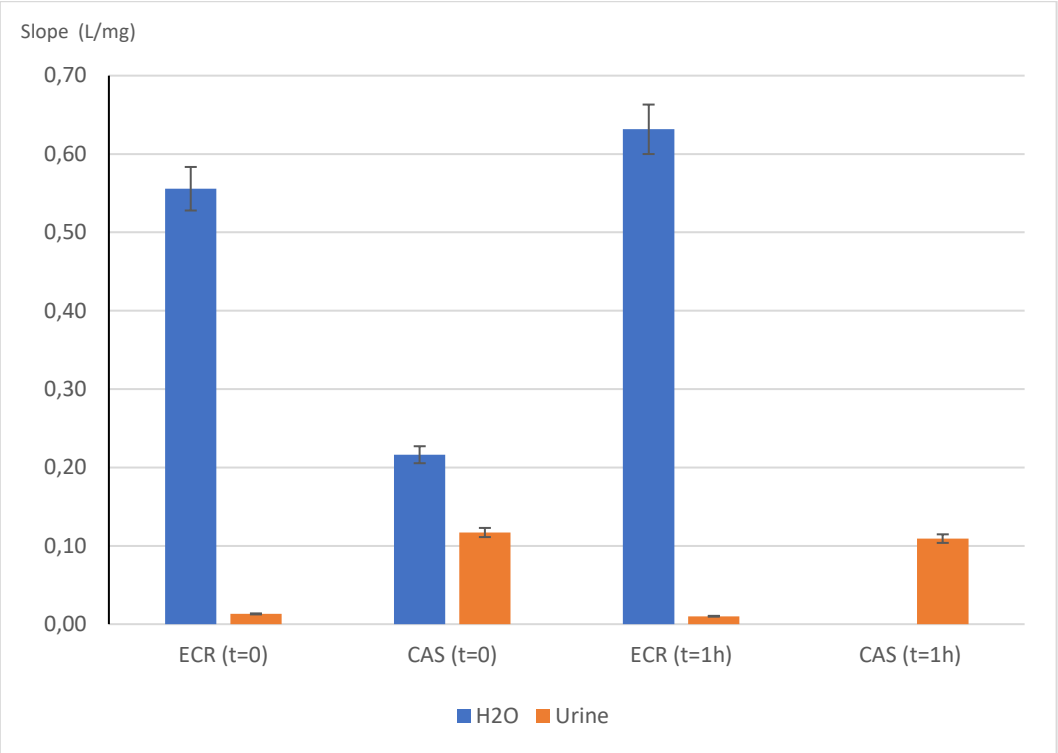

Figure 6 - Reagent Selection: calibration curve slopes of reagents in water and synthetic urine standard solutions; Measurement Times: 0 and 1 hour; Comparison of CAS and ECR kinetics.

3.2. Matrix Influence - batch studies

The previous results indicate that the urine matrix adversely affects aluminium determination, resulting in lower sensitivity compared to measurements taken in a water matrix. To enhance sensitivity, an investigation was conducted into the influence of urine components on aluminium quantification. The composition of urine includes various substances that can complicate the analysis of metal concentrations within this matrix.

Recognising these interferences is essential for improving the accuracy and reliability of aluminium measurements in urine samples.

Following some research, prompted by inconclusive results, it was found that the urine matrix contains components that react with aluminium, thus interfering with its measurement [61, 1, 62, 31, 63]. In this study, base urine, defined as urine devoid of interfering components, was compared to complete urine to assess sensitivity in quantification. The results clearly indicated that the presence of interfering substances in complete urine adversely affected measurement accuracy. To address this issue, the components were separated to identify the optimal matrix composition for accurately determining aluminium concentration. Initially, the influence of lactic acid was tested, followed by an evaluation of uric acid interference and their combination with the base matrix. Lactic acid demonstrated a more significant negative impact on quantification compared to uric acid. The combination of the base matrix with both acids yielded sensitivity values that fell between those observed for each acid individually. Despite these interferences, sensitivity remained higher when compared to tests conducted with complete urine, suggesting that neither lactic acid nor uric acid was the primary cause of measurement interference. Subsequently, the influence of sulphates (magnesium and sodium) was investigated, revealing higher sensitivity values than those observed with the acids. Consequently, quantification was performed using the base matrix combined with both lactic and uric acids alongside magnesium and sodium sulphates. This combination achieved sensitivity levels exceeding those obtained with tests conducted solely on the base matrix.

Next, phosphates were tested and identified as significant interferents, exhibiting markedly reduced sensitivity values even when diluted by half. The hypothesis that substituting sodium and magnesium sulphates with nitrates (sodium and magnesium) could reduce measurement errors was also explored; however, these nitrates yielded less satisfactory results compared to sulphates when measured individually. Finally, both nitrates from the previous tests and phosphates present in complete urine (potassium dihydrogen phosphate and dipotassium hydrogen phosphate) were added to the base matrix. This comprehensive series of tests concluded that potassium dihydrogen phosphate and dipotassium hydrogen phosphate were the principal interferents in determining aluminium concentration, as sensitivity values obtained from this combination approached zero. The following graph (Fig. 7) illustrates the sensitivity observed across each set of tests with different component combinations.

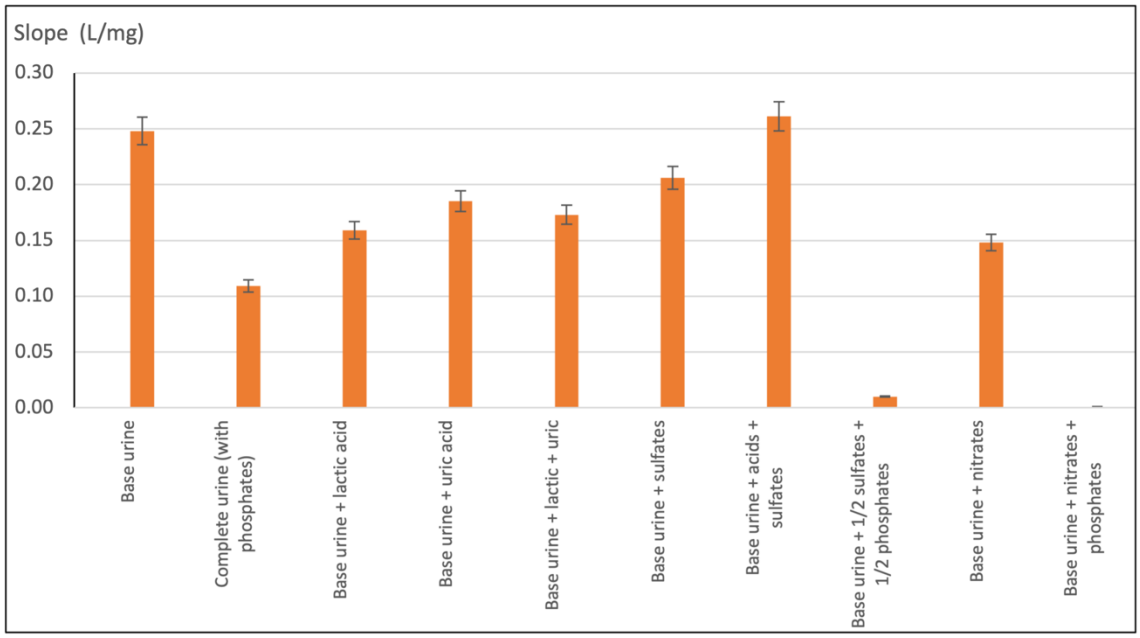

Figure 7 - Urine matrix influence in aluminium concentration measurement.

In this figure, it is evident that the complete matrix exhibits lower sensitivity compared to the base urine matrix. It can also be concluded that, among all combinations of various components with the base matrix, the one demonstrating the highest sensitivity is the mixture containing the base matrix, lactic acid, uric acid, sodium sulphate, and magnesium sulphate.

3.3. Detection area - paper studies

In the initial phase of this study, the microfluidic paper-based analytical device (µPAD) was assembled using two layers of paper: the upper layer comprised Whatman 1 (W1) paper, while the lower layer utilized Whatman 3 (W3) paper. The upper layer contained 15 µL of a color reagent, and the lower layer served as a reservoir with an empty disc.

To determine the most suitable detection area for aluminum measurement, three different areas were examined: the center of the disc, the entire disc, and the entire reverse side of the disc. The findings indicated that for quantifying aluminum in urine, measuring the total area of the reverse side of each disc was optimal. This conclusion was based on the observation that the intensity of coloration on the discs was significantly greater on the reverse side compared to both the center and the entire front side.

Consequently, it became necessary to modify the configuration of the µPAD. The new arrangement involved reversing the positions of the discs; thus, W1 and W3 exchanged their placements. In this updated design, W1, which contained the color reagent, was positioned beneath W3. This adjustment enabled us to achieve the desired results, as illustrated in the accompanying figures.

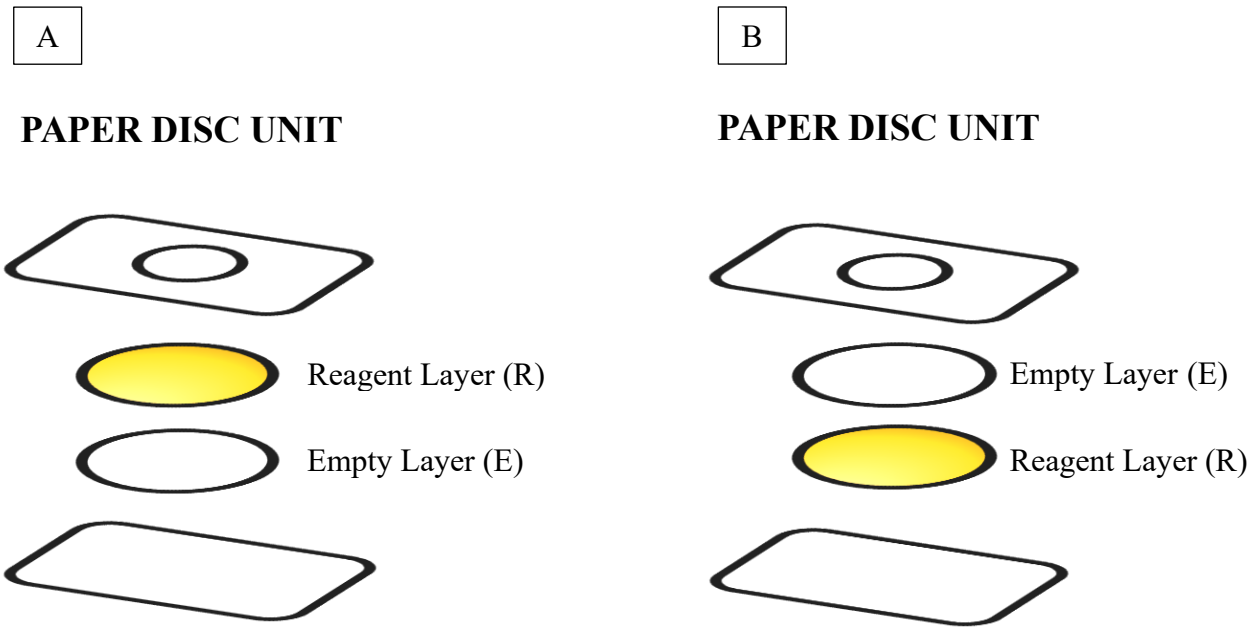

Figure 8 - (A)First µPAD layers configuration. (B) Final configuration of µPAD layers.

3.4. Reaction time - paper studies

The device's response time was assessed using a range of biological specimens. Measurements were recorded at 10-minute intervals over a total period of 30 minutes. The resultant graph demonstrates that the optimal time point for quantifying aluminum concentration in urine samples is 20 minutes after initiating the test. At this time point, sensitivity is greater than that observed at 10 minutes, while not significantly differing from the measurement taken at 30 minutes. Therefore, to achieve results with the highest degree of sensitivity, it is recommended that measurements be conducted 20 minutes after the solution is injected into the various reaction wells of the device.
The next graph shows the kinetics test in the device.

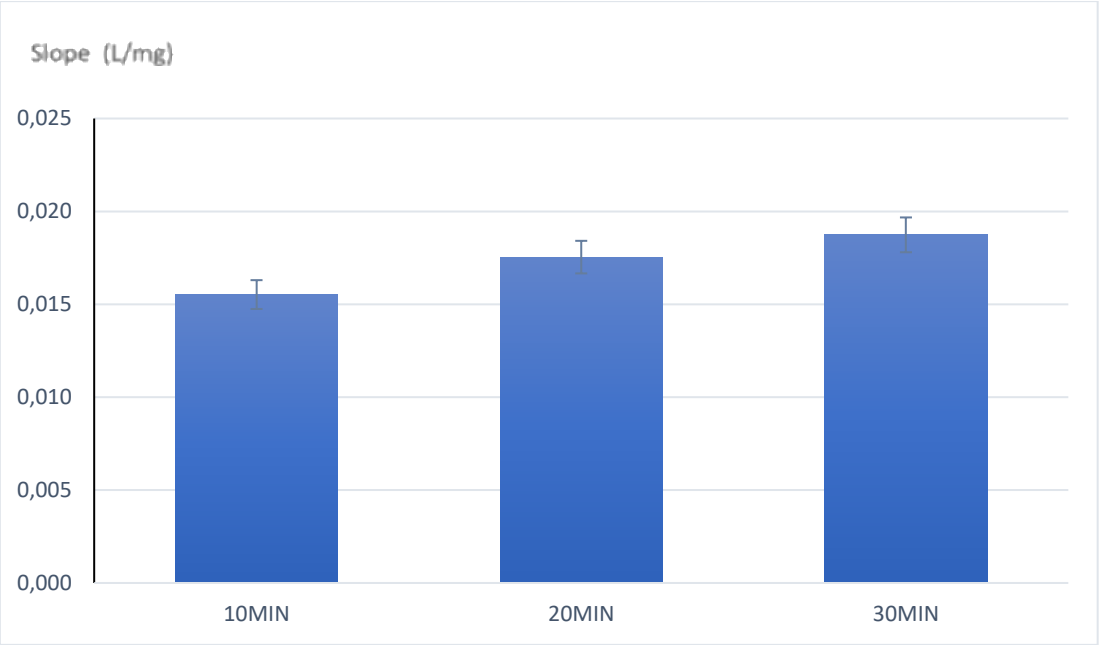

Figure 9 – Reaction time studies: optimization of μPAD scanning time through slope analysis.

3.5. Analytical features of the developed paper device

After establishing the operational parameters for the determination of aluminium in a microfluidic device, the features of the developed μPAD are summarised in the table below (Table I).

Following the extensive studies conducted to determine aluminium concentrations using the coloured complex formed with chromogenic agent CAS, the analytical characteristics of the developed microfluidic paper-based analytical device are summarized in Table I. The limit of detection (LOD) and the limit of quantification (LOQ) were established in accordance with the recommendations of the International Union of Pure and Applied Chemistry (IUPAC). The LOD was calculated as three times the standard deviation of the intercept (n=5), while the LOQ was determined ten times this value, divided by the average slope of the calibration curves (n=5). The LOD was evaluated across various scanning durations, ranging from 10 to 30 minutes (see Fig. 9). The results indicated that the lowest LOD values were consistently achieved between scanning times of 20 to 30 minutes. Consequently, in was determined that the device should be scanned for a duration of 30 minutes following sample insertion. The dynamic range of the method was established based on the LOQ obtained, providing a robust range for quantifying aluminium in urine samples. The repeatability of the μPAD was assessed by calculating the relative standard deviation (RSD) from five calibration curves.

Table I - Summary of features for the developed μPAD: LOD, limit of detection; LOQ, limit of quantification; RSD, recovery standard deviation (%).

Dynamic range	0.40 - 2.0 mg/L
Typical calibration curve (A = Slope ± SD × $[Al^{3+}]$ + intercept ± SD)	A=0.020 ± 0.002 × $[Al^{3+}]$ + 0.007 ± 0.002
LOD (mg/L)	0.12
LOQ (mg/L)	0.40
RSD (n=5)	9.7%
Reagent consumption per μPAD (mg)	• CAS ($C_{23}H_{13}Cl_2Na_3O_9S$): 0.36 mg • $NaO_2CCH_3 \cdot 3H_2O$: 24.48 mg • CH_3COOH: 0.43 mg
Sample consumption per determination (μL)	360

3.6. Application to urine samples

Table II presents the results of the recovery studies conducted to assess the performance and reliability of the developed analytical method. This table summarises the percentage recovery of aluminium from various samples, highlighting the effectiveness of the method under different conditions. The data included will provide insight into the accuracy and precision of the measurements obtained, which are crucial for validating the analytical technique employed in this research.

Table II - Recovery study results for aluminium detection in biological samples.

Sample ID	$[Al^{3+}]$ initial (mg/L)	SD (mg/L)	$[Al^{3+}]$ added (mg/L)	$[Al^{3+}]$ found (mg/L)	SD (mg/L)	Recovery %
S1	< LOQ	-	0.455	0.478	0.029	105
			1.15	1.19	0.095	103
S2			0.455	0.462	0.152	102
			1.15	1.27	0.293	110
S3			0.455	0.457	0.035	101
			1.15	1.21	0.038	104
S4	0.570*	0.083	0.700	1.446	0.214	125
S5	0.944*	0.068		1.912	0.150	138
S6	0.819*	0.160		1.508	0.072	98.4
S7	0.145*	0.072		0.846	0.332	100

* Concentration values obtained recuring to spiked values-

4. Conclusion

The utilisation of μPADs for aluminium quantification in urine samples presents an efficient strategy for assessing aluminium burden in individuals at risk of Alzheimer's disease. This method is advantageous due to its simplicity, minimal reagent requirements, and capacity to yield quantifiable results without extensive laboratory infrastructure. Furthermore, μPAD technology is adaptable for detecting specific chelation reactions between aluminium ions and reagents impregnated in the paper, resulting in a colorimetric or fluorescent signal that correlates with aluminium concentration.

This thesis has explored the role of a new aluminium measurement device, which functions as a potential tool for detecting and quantifying harmful concentrations related to the pathogenesis of Alzheimer's disease (AD). This investigation highlights the link between elevated aluminium levels and neurotoxicity, which may contribute to cognitive decline. The need for reliable biomarkers and diagnostic methods for assessing aluminium exposure is critical for the early detection and risk assessment of Alzheimer's. One promising approach identified is the quantification of urinary aluminium (uAl), as urinary levels can reflect recent exposure and provide valuable insights into aluminium's association with AD.

The application of μPADs involves critical steps such as sample preparation, reagent selection, and calibration. Reagents like Chrome Azurol S (CAS) demonstrate high specificity for Al^{3+} ions, facilitating selective complexation that produces measurable optical signals. Coupling μPADs with image analysis software enhances sensitivity and allows precise quantification of aluminium levels. Studies have shown that μPADs can detect uAl levels in the low micromolar range, which is significant for identifying individuals exposed to elevated sources of aluminium. These findings indicate that μPAD-based uAl quantification could serve as a non-invasive, rapid screening tool to support Alzheimer's diagnosis, particularly in environmental or occupational contexts where aluminium exposure is a concern.

Despite these advancements, Alzheimer's disease remains a significant global health challenge, with traditional diagnostic techniques often lacking sensitivity and specificity, especially in the early stages. Cognitive assessments may fail to capture subtle cognitive declines adequately, while neuroimaging techniques can be costly and require significant expertise for accurate interpretation[50]. Existing biomarkers frequently do not provide timely or precise diagnoses due to their retrospective nature, primarily relying on post-mortem analyses that do not allow for prospective evaluations of disease progression[51].

Recent studies suggest that machine learning (ML) approaches could enhance early detection by integrating multimodal datasets, including genetic information and neuroimaging[52]. However, challenges regarding generalisability and interpretability remain and must be addressed to improve clinical utility[52]. Furthermore, the detection of aluminium in biological systems poses significant challenges due to limitations in current sensing technologies. Existing methods often rely on complex instrumentation that is expensive and requires specialised training to operate effectively[53]. While optical probes show promise due to their sensitivity and rapid response times, issues related to selectivity

and operational complexity persist[53]. Traditional detection methods also suffer from high rates of false positives and negatives within complex biological matrices where aluminium concentrations can vary significantly[54].

To address these challenges, recent advancements in deep learning methodologies have begun improving defect detection accuracy in industrial applications; however, similar innovations are needed within biological contexts[55]. Integrating advanced computational techniques with novel sensor designs could lead to more effective aluminium detection methods that are both reliable and user-friendly.

In summary, this research aims to bridge existing gaps by developing a novel diagnostic framework that leverages machine learning algorithms alongside innovative sensor technologies. By focusing on multimodal data integration for AD diagnosis and enhancing the sensitivity and specificity of aluminium detection methods through novel optical sensors, this work seeks to provide comprehensive solutions addressing current shortcomings. The implications extend beyond academic inquiry; they hold substantial promise for improving clinical outcomes. Enhanced diagnostic accuracy could facilitate earlier interventions for Alzheimer's disease, ultimately improving patient quality of life and reducing healthcare costs associated with late-stage care[50]. Additionally, more reliable methods for detecting aluminium could enable better monitoring of environmental exposure and its associated health risks, particularly among vulnerable populations[53].

References

(1) Sarigul, N.; Korkmaz, F.; Kurultak, İ. A New Artificial Urine Protocol to Better Imitate Human Urine. *Sci. Rep.* **2019**, *9*, 20159. https://doi.org/10.1038/s41598-019-56693-4.

(2) Kalantari, S.; Jafari, A.; Moradpoor, R.; Ghasemi, E.; Khalkhal, E. Human Urine Proteomics: Analytical Techniques and Clinical Applications in Renal Diseases. *Int. J. Proteomics* **2015**, *2015*, 782798. https://doi.org/10.1155/2015/782798.

(3) *Urinalysis - Mayo Clinic*. https://www.mayoclinic.org/tests-procedures/urinalysis/about/pac-20384907 (accessed 2024-11-25).

(4) *Urinalysis: What Is It, Testing, Indications*. Osmosis. https://www.osmosis.org/answers/urinalysis (accessed 2024-11-25).

(5) Echeverry, G.; Hortin, G. L.; Rai, A. J. Introduction to Urinalysis: Historical Perspectives and Clinical Application. *Methods Mol. Biol. Clifton NJ* **2010**, *641*, 1–12. https://doi.org/10.1007/978-1-60761-711-2_1.

(6) *Aluminum | ToxFAQs™ | ATSDR*. https://wwwn.cdc.gov/TSP/ToxFAQs/ToxFAQsDetails.aspx?faqid=190&toxid=34# (accessed 2024-10-22).

(7) Sable, H.; Singh, V.; Kumar, V.; Roy, A.; Pandit, S.; Kaur, K.; Rustagi, S.; Malik, S. Toxicological and Bioremediation Profiling of Nonessential Heavy Metals (Mercury, Chromium, Cadmium, Aluminium) and Their Impact on Human Health: A Review. *Toxicol. Anal. Clin.* **2024**, *36* (3), 205–234. https://doi.org/10.1016/j.toxac.2024.03.096.

(8) Willhite, C. C.; Karyakina, N. A.; Yokel, R. A.; Yenugadhati, N.; Wisniewski, T. M.; Arnold, I. M. F.; Momoli, F.; Krewski, D. Systematic Review of Potential Health Risks Posed by Pharmaceutical, Occupational and Consumer Exposures to Metallic and Nanoscale Aluminum, Aluminum Oxides, Aluminum Hydroxide and Its Soluble Salts. *Crit. Rev. Toxicol.* **2014**, *44* (Suppl 4), 1. https://doi.org/10.3109/10408444.2014.934439.

(9) Martín, S. P. S.; Bauçà, J. M.; Martinez-Morillo, E. Determination of Aluminum Concentrations in Biological Specimens: Application in the Clinical Laboratory. *Adv. Lab. Med.* **2022**, *3* (2), 153. https://doi.org/10.1515/almed-2022-0056.

(10) Tp22-C2.Pdf. https://www.atsdr.cdc.gov/toxprofiles/tp22-c2.pdf (accessed 2024-10-28).

(11) Campbell, A. The Potential Role of Aluminium in Alzheimer's Disease. *Nephrol. Dial. Transplant.* **2002**, *17* (suppl_2), 17–20. https://doi.org/10.1093/ndt/17.suppl_2.17.

(12) Belojević, G.; Jakovljević, B. [Aluminum and Alzheimer's disease]. *Srp. Arh. Celok. Lek.* **1998**, *126* (7–8), 283–289.

(13) Kawahara, M.; Kato-Negishi, M. Link between Aluminum and the Pathogenesis of Alzheimer's Disease: The Integration of the Aluminum and Amyloid Cascade Hypotheses. *Int. J. Alzheimers Dis.* **2011**, *2011*, 276393. https://doi.org/10.4061/2011/276393.

(14) Choudhury, R.; Ashtekar, H.; Khot, K. B.; Malngiang, M.; Kumar, M. V.; Mandal, S.; Das, B. Aluminum Toxicity Induced Alzheimer's Disease and Its

Potential Treatment Using Antioxidants - a Review. *Braz. J. Pharm. Sci.* **2023**, *59*, e21587. https://doi.org/10.1590/s2175-97902023e21587.
(15) Huat, T. J.; Camats-Perna, J.; Newcombe, E. A.; Valmas, N.; Kitazawa, M.; Medeiros, R. Metal Toxicity Links to Alzheimer's Disease and Neuroinflammation. *J. Mol. Biol.* **2019**, *431* (9), 1843–1868. https://doi.org/10.1016/j.jmb.2019.01.018.
(16) *Alzheimer's disease and aluminum: Is there a connection?* https://www.medicalnewstoday.com/articles/does-aluminum-cause-alzheimers (accessed 2024-11-18).
(17) Aguiar, J. I. S.; Silva, M. T. S.; Ferreira, H. A. G.; Pinto, E. C. B.; Vasconcelos, M. W.; Rangel, A. O. S. S.; Mesquita, R. B. R. Development of a Microfluidic Paper-Based Analytical Device for Magnesium Determination in Saliva Samples. *Talanta Open* **2022**, *6*, 100135. https://doi.org/10.1016/j.talo.2022.100135.
(18) Iaccarino, L.; Burnham, S. C.; Dell'Agnello, G.; Dowsett, S. A.; Epelbaum, S. Diagnostic Biomarkers of Amyloid and Tau Pathology in Alzheimer's Disease: An Overview of Tests for Clinical Practice in the United States and Europe. *J. Prev. Alzheimers Dis.* **2023**, *10* (3), 426–442. https://doi.org/10.14283/jpad.2023.43.
(19) Busche, M. A.; Hyman, B. T. Synergy between Amyloid-β and Tau in Alzheimer's Disease. *Nat. Neurosci.* **2020**, *23* (10), 1183–1193. https://doi.org/10.1038/s41593-020-0687-6.
(20) Bloom, G. S. Amyloid-β and Tau: The Trigger and Bullet in Alzheimer Disease Pathogenesis. *JAMA Neurol.* **2014**, *71* (4), 505–508. https://doi.org/10.1001/jamaneurol.2013.5847.
(21) *Amyloid and tau: the proteins involved in dementia*. https://www.dementiasplatform.uk/news-and-media/blog/amyloid-and-tau-the-proteins-involved-in-dementia (accessed 2024-11-18).
(22) Pradeepkiran, J. A.; Baig, J.; Islam, M. A.; Kshirsagar, S.; Reddy, P. H. Amyloid-β and Phosphorylated Tau Are the Key Biomarkers and Predictors of Alzheimer's Disease. *Aging Dis.* **2024**, 0. https://doi.org/10.14336/AD.2024.0286.
(23) Zhang, Y.; Chen, H.; Li, R.; Sterling, K.; Song, W. Amyloid β-Based Therapy for Alzheimer's Disease: Challenges, Successes and Future. *Signal Transduct. Target. Ther.* **2023**, *8* (1), 1–26. https://doi.org/10.1038/s41392-023-01484-7.
(24) *Atomic Absorption Spectrometry (AAS) Information - PT*. https://www.thermofisher.com/uk/en/home/industrial/spectroscopy-elemental-isotope-analysis/spectroscopy-elemental-isotope-analysis-learning-center/trace-elemental-analysis-tea-information/atomic-absorption-aa-information.html (accessed 2024-11-06).
(25) *ICP-OES vs ICP-MS, ICP-OES metals analysis | Agilent*. https://www.agilent.com/en/product/atomic-spectroscopy/icp-oes-vs-icp-ms?gad_source=1&gbraid=0AAAADSHcWchF2YW0Pn-33iI0NOL71kO_&gclid=CjwKCAiAxKy5BhBbEiwAYiW--5mz5fD8YAscbWbFnk9g64drSA5ITXO-DihPdkQqdftJFhbL0YPSVhoCGS8QAvD_BwE&gclsrc=aw.ds (accessed 2024-11-07).
(26) *Atomic Absorption Spectrophotometer Pros and Cons*. Buck Scientific Instruments LLC. https://www.bucksci.com/pages/atomic-absorption-spectrophotometer (accessed 2024-11-06).
(27) *Industry ApplicationAdvantages and disadvantages of inductively coupled plasma atomic em ission spect_新闻中心_Beijing Huake Yitong Analytical Instrument Co., Ltd.* http://www.bjhkyt.com/newsen27.html (accessed 2024-11-07).

(28) Soda, Y.; Robinson, K. J.; Cherubini, T. J.; Bakker, E. Colorimetric Absorbance Mapping and Quantitation on Paper-Based Analytical Devices. *Lab. Chip* **2020**, *20* (8), 1441–1448. https://doi.org/10.1039/D0LC00028K.
(29) Morbioli, G. G.; Mazzu-Nascimento, T.; Stockton, A. M.; Carrilho, E. Technical Aspects and Challenges of Colorimetric Detection with Microfluidic Paper-Based Analytical Devices (μPADs) - A Review. *Anal. Chim. Acta* **2017**, *970*, 1–22. https://doi.org/10.1016/j.aca.2017.03.037.
(30) Peters, K. L.; Corbin, I.; Kaufman, L. M.; Zreibe, K.; Blanes, L.; McCord, B. R. Simultaneous Colorimetric Detection of Improvised Explosive Compounds Using Microfluidic Paper-Based Analytical Devices (μPADs). *Anal. Methods* **2014**, *7* (1), 63–70. https://doi.org/10.1039/C4AY01677G.
(31) Melo, M. M. P.; Machado, A.; Rangel, A. O. S. S.; Mesquita, R. B. R. Disposable Microfluidic Paper-Based Device for On-Site Quantification of Urinary Creatinine. *Chemosensors* **2023**, *11* (7), 368. https://doi.org/10.3390/chemosensors11070368.
(32) Ferreira, F. T. S. M.; Mesquita, R. B. R.; Rangel, A. O. S. S. Novel Microfluidic Paper-Based Analytical Devices (μPADs) for the Determination of Nitrate and Nitrite in Human Saliva. *Talanta* **2020**, *219*, 121183. https://doi.org/10.1016/j.talanta.2020.121183.
(33) Lam, T.; Devadhasan, J. P.; Howse, R.; Kim, J. A Chemically Patterned Microfluidic Paper-Based Analytical Device (C-μPAD) for Point-of-Care Diagnostics. *Sci. Rep.* **2017**, *7* (1), 1188. https://doi.org/10.1038/s41598-017-01343-w.
(34) Dortez, S.; Pacheco, M.; Gasull, T.; Crevillen, A. G.; Escarpa, A. A Dual Colorimetric-Electrochemical Microfluidic Paper-Based Analytical Device for Point-of-Care Testing of Ischemic Strokes. *Lab. Chip* **2024**, *24* (18), 4253–4263. https://doi.org/10.1039/D4LC00398E.
(35) McCracken, K. E.; Angus, S. V.; Reynolds, K. A.; Yoon, J.-Y. Multimodal Imaging and Lighting Bias Correction for Improved μPAD-Based Water Quality Monitoring via Smartphones. *Sci. Rep.* **2016**, *6* (1), 27529. https://doi.org/10.1038/srep27529.
(36) *Fabrication of a paper-based facile and low-cost microfluidic device and digital imaging technique for point-of-need monitoring of hypochlorite - PubMed*. https://pubmed.ncbi.nlm.nih.gov/37486009/ (accessed 2024-11-18).
(37) Soman, S. S.; Samad, S. A.; Venugopalan, P.; Kumawat, N.; Kumar, S. Microfluidic Paper Analytic Device (μPAD) Technology for Food Safety Applications. *Biomicrofluidics* **2024**, *18* (3), 031501. https://doi.org/10.1063/5.0192295.
(38) Charernchai, S.; Chikae, M.; Phan, T. T.; Wonsawat, W.; Hirose, D.; Takamura, Y. Automated Paper-Based Femtogram Sensing Device for Competitive Enzyme-Linked Immunosorbent Assay of Aflatoxin B1 Using Submicroliter Samples. *Anal. Chem.* **2022**, *94* (12), 5099. https://doi.org/10.1021/acs.analchem.1c05401.
(39) *Recent Advances of Fluid Manipulation Technologies in Microfluidic Paper-Based Analytical Devices (μPADs) toward Multi-Step Assays*. https://www.mdpi.com/2072-666X/11/3/269 (accessed 2024-11-18).
(40) *Aluminum Urine Test: Procedure, Purpose, Results, Normal levels, Cost, Price, Online booking*. myUpchar. https://www.myupchar.com/en/test/aluminum-urine (accessed 2024-11-21).

(41) *Aluminium - 24 Hours Urine Test Price, Normal Range | Sprint Diagnostics Hyderabad*. https://www.sprintdiagnostics.in/hyderabad/test/aluminium-24-hours-urine (accessed 2024-11-21).
(42) Dispinar, D. Determination of Metal Quality of Aluminium and Its Alloys.
(43) *Aluminium - Urine | GetLabTest.com*. https://www.getlabtest.com/lab-test/aluminium-urine (accessed 2024-11-21).
(44) Ruha, A.-M. Recommendations for Provoked Challenge Urine Testing. *J. Med. Toxicol. Off. J. Am. Coll. Med. Toxicol.* **2013**, *9* (4), 318–325. https://doi.org/10.1007/s13181-013-0350-7.
(45) Crinnion, W. J. The Benefits of Pre- and Post-Challenge Urine Heavy Metal Testing: Part 1. *Altern. Med. Rev. J. Clin. Ther.* **2009**, *14* (1), 3–8.
(46) Abdollahi-Aghdam, A.; Majidi, M. R.; Omidi, Y. Microfluidic Paper-Based Analytical Devices (μPADs) for Fast and Ultrasensitive Sensing of Biomarkers and Monitoring of Diseases. *BioImpacts BI* **2018**, *8* (4), 237–240. https://doi.org/10.15171/bi.2018.26.
(47) Noviana, E.; Ozer, T.; Carrell, C. S.; Link, J. S.; McMahon, C.; Jang, I.; Henry, C. S. Microfluidic Paper-Based Analytical Devices: From Design to Applications. *Chem. Rev.* **2021**, *121* (19), 11835–11885. https://doi.org/10.1021/acs.chemrev.0c01335.
(48) Ghosh, R.; Gopalakrishnan, S.; Savitha, R.; Renganathan, T.; Pushpavanam, S. Fabrication of Laser Printed Microfluidic Paper-Based Analytical Devices (LP-μPADs) for Point-of-Care Applications. *Sci. Rep.* **2019**, *9* (1), 7896. https://doi.org/10.1038/s41598-019-44455-1.
(49) Boobphahom, S.; Nguyet Ly, M.; Soum, V.; Pyun, N.; Kwon, O.-S.; Rodthongkum, N.; Shin, K. Recent Advances in Microfluidic Paper-Based Analytical Devices toward High-Throughput Screening. *Molecules* **2020**, *25* (13), 2970. https://doi.org/10.3390/molecules25132970.
(50) *New scanning technology could help diagnose Alzheimer's disease using light*. https://www.research.va.gov/currents/0721-New-scanning-technology-could-help-diagnose-Alzheimers-disease-using-light.cfm (accessed 2024-11-24).
(51) Authors; Wells, C.; Horton, J. *An Overview of New and Emerging Technologies for Early Diagnosis of Alzheimer Disease: Emerging Health Technologies*; CADTH Horizon Scans; Canadian Agency for Drugs and Technologies in Health: Ottawa (ON), 2022.
(52) Lott, S. A.; Streel, E.; Bachman, S. L.; Bode, K.; Dyer, J.; Fitzer-Attas, C.; Goldsack, J. C.; Hake, A.; Jannati, A.; Fuertes, R. S.; Fromy, P. Digital Health Technologies for Alzheimer's Disease and Related Dementias: Initial Results from a Landscape Analysis and Community Collaborative Effort. *J. Prev. Alzheimers Dis.* **2024**, *11* (5), 1480–1489. https://doi.org/10.14283/jpad.2024.103.
(53) Https://Pmc.Ncbi.Nlm.Nih.Gov/Articles/PMC10565909/.
(54) Zhang, Y.; Bi, K.; Zhou, L.; Wang, J.; Huang, L.; Sun, Y.; Peng, G.; Wu, W. Advances in Blood Biomarkers for Alzheimer’s Disease: Ultra-Sensitive Detection Technologies and Impact on Clinical Diagnosis. *Degener. Neurol. Neuromuscul. Dis.* **2024**, *14*, 85–102. https://doi.org/10.2147/DNND.S471174.
(55) *Research on a new diagnostic tool for Alzheimer's disease presented at the 30th Annual Alzheimer Day at Northwestern University, Chicago*. Irish Research Council. https://research.ie/what-we-do/loveirishresearch/blog/research-on-a-new-diagnostic-tool-for-alzheimers-disease-presented-at-the-30th-annual-alzheimer-day-at-northwestern-university-chicago/ (accessed 2024-11-24).

(56) Brooks, T.; Keevil, C. W. A Simple Artificial Urine for the Growth of Urinary Pathogens. *Lett. Appl. Microbiol.* **1997**, *24* (3), 203–206. https://doi.org/10.1046/j.1472-765x.1997.00378.x.
(57) Zolotov, Y. Marczenko, Z. and Balcerzak, M., Separation, Preconcentration, and Spectrophotometry in Inorganic Analysis, Amsterdam: Elsevier, 2001, 528 Pp. *J. Anal. Chem. - J ANAL CHEM-ENGL TR* **2002**, *57*, 366–367. https://doi.org/10.1023/A:1014918902386.
(58) Burden, T. J.; Whitehead, M. W.; Thompson, R. P. H.; Powell, J. J. Preparation of Urine Samples for Trace Metal Determination: A Study with Aluminium Analysis by Inductively Coupled Plasma Optical Emission Spectrometry. *Ann. Clin. Biochem.* **1998**, *35* (2), 245–253. https://doi.org/10.1177/000456329803500208.
(59) Mesquita, R. B. R.; Rangel, A. O. S. S. Development of Sequential Injection Methodologies for the Spectrophotometry Direct and Kinetic Determination of Aluminium in Natural and Waste Waters. *J. Braz. Chem. Soc.* **2008**, *19* (6), 1171–1179. https://doi.org/10.1590/S0103-50532008000600018.
(60) Bouzid, B.; Macdonald, A. M. G. Flow-Injection Spectrophotometric Determination of Aluminium Based on Chrome Azurol S and Cetylpyridinium Chloride. *Anal. Chim. Acta* **1988**, *207*, 337–342. https://doi.org/10.1016/S0003-2670(00)80811-9.
(61) Prywer, J.; Kozanecki, M.; Mielniczek-Brzóska, E.; Torzewska, A. Solid Phases Precipitating in Artificial Urine in the Absence and Presence of Bacteria Proteus Mirabilis—A Contribution to the Understanding of Infectious Urinary Stone Formation. *Crystals* **2018**, *8* (4), 164. https://doi.org/10.3390/cryst8040164.
(62) Takis, P. G.; Schäfer, H.; Spraul, M.; Luchinat, C. Deconvoluting Interrelationships between Concentrations and Chemical Shifts in Urine Provides a Powerful Analysis Tool. *Nat. Commun.* **2017**, *8* (1), 1662. https://doi.org/10.1038/s41467-017-01587-0.
(63) Biga, L. M.; Bronson, S.; Dawson, S.; Harwell, A.; Hopkins, R.; Kaufmann, J.; LeMaster, M.; Matern, P.; Morrison-Graham, K.; Oja, K.; Quick, D.; Runyeon, J.; Oeru, O.; OpenStax. 26.4 Acid-Base Balance. **2019**.