A DISSERTATION ON

Quantitative estimation of Total Amino Acids in blood serum samples using Ninhydrin Paper based Microfluidics and a digital scanner

SUBMITTED TO THE

DEPARTMENT OF BIOENGINEERING
FACULTY OF ENGINEERING & INFORMATION TECHNOLOGY
INTEGRAL UNIVERSITY, LUCKNOW

IN PARTIAL FULFILMENT
FOR THE
DEGREE OF MASTER OF TECHNOLOGY IN BIOTECHNOLOGY

BY
PRIYA AGRAWAL
M.Tech Biotechnology (IV Semester)
Roll No: 2101361008
UNDER THE SUPERVISION OF
Dr. PUNIT KUMAR SINGH
Assistant Professor
Integral University, Lucknow

INTEGRAL UNIVERSITY, DASAULI, KURSI ROAD
LUCKNOW- 226026

DECLARATION FORM

I, **Priya Agarwal** , a student of **M.tech Biotechnology** (2^{nd} Year/ 4^{th} Semester), Integral University have completed my six months dissertation work entitled **"Quantitative estimation of Total Amino Acids in blood serum samples using Ninhydrin Paper based Microfluidics and a digital scanner"** successfully from **Integral University and KGMU** under the able guidance of **Dr. Punit Kumar Singh.**

I, hereby, affirm that the work has been done by me in all aspects. I have sincerely prepared this project report and the results reported in this study are genuine and authentic.

Name and Signature of Student with Date

Name and Signature of Course Coordinator with Date

INTEGRAL UNIVERSITY
LUCKNOW - INDIA
Phone No.: +91(0522) 2890812, 2890730, 3296117, 6451039, Fax No.: 0522-2890809
Kursi Road, Lucknow-226026 Uttar Pradesh (INDIA)

CERTIFICATE

This is certify that Ms. **Priya Agrawal** (Enrollment Number 2101361008) has carried out the research work presented in this thesis entitled **"Quantitative estimation of total amino acid in blood serum sample using ninhydrin paper based microfluidics and a digital scanner"** for the award of **M.Tech Biotechnology** from Integral University, Lucknow under **Dr.Punit Kumar Singh** supervision. The thesis embodies results of original work and studies carried out by the student himself and the contents of the thesis do not form the basis for the award of any other degree to the candidate or to anybody else from this or any other University/Institution. The dissertation was a compulsory part of her **M.Tech Biotechnlogy** degree.

I wish her good luck and bright future.

Dr. Punit Kumar Singh
Assistant Professor
Department of Bioengineering

INTEGRAL UNIVERSITY
LUCKNOW - INDIA
Phone No.: +91(0522) 2890812, 2890730, 3296117, 6451039, Fax No.: 0522-2890809
Kursi Road, Lucknow-226026 Uttar Pradesh (INDIA)

CERTIFICATE BY INTERNAL ADVISOR

This is to certify that **Priya Agrawal** a student of **M.Tech Biotechnology** (2nd Year/4th Semester), Integral University has completed her six months dissertation work entitled **"Quantitative estimation of total amino acid in blood serum sample using ninhydrin paper based microfluidics and a digital scanner"** successfully. She has completed this work from Integral University and KGMU under the guidance of Dr.Punit Kumar Singh . The dissertation was a compulsory part of her **M.Tech Biotechnology** degree.

I wish her good luck and bright future.

Dr. Khwaja Osama
Assistant Professor
Department of Bioengineering
Faculty of Engineering & Information Technology

INTEGRAL UNIVERSITY
LUCKNOW - INDIA
Phone No.: +91(0522) 2890812, 2890730, 3296117, 6451039, Fax No.: 0522-2890809
Kursi Road, Lucknow-226026 Uttar Pradesh (INDIA)

TO WHOM IT MAY CONCERN

This is to certify that **Priya Agrawal ,** a student of **M.Tech Biotechnology** (2nd Year/4th Semester), Integral University has completed her six months dissertation work entitled **"Quantitative estimation of total amnio acid in blood serum sample using ninhydrin paper based microfluidics and a digital scanner"** successfully. She has completed this work from Integral University and KGMU under the guidance of Dr.Punit Kumar Singh The dissertation was a compulsory part of her **M.Tech Biotechnology** degree.

I wish her good luck and bright future.

Dr. Alvina Farooqui
Professor and Head
Department of Bioengineering
Faculty of Engineering & Information Technology

ACKNOWLEDGMENT

First of all, I bow in reverence to the Almighty for blessing me with strong will power, patience and confidence, which helped me in completing the present work.

At the very outset I pay my warm thanks to our Honorable **Chancellor and Founder, Integral University, Lucknow. Prof. S.W Akhtar** for providing excellent infrastructure and Lab facilities at IIRC-4 Integral University, Lucknow. I am also grateful to Honorable **Vice Chancellor Integral University, Lucknow. Prof. JavedMusarrat** for his continuous motivation and a Special vote of thanks to **Pro Chancellor, Dr. NadeemAkhtar** for his encouragement and support,I would also like to extend my gratitude to **Pro vice chancellor, Professor Aquil Ahmed** for creating a humble and peaceful environment.

I would like to thank the **Dean, Faculty of Engineering and Information Technology, Professor T. Usmani**and I would like to express my special thanks to **Dr. Alvinafarooqui (Head, Department of Bioengineering)** for given me an opportunity to join the department laboratory and providing all the necessary facilities ever since I started my work.

I would I like to express my deep sense of gratitude to internal advisor and supervisor **Dr.Punit Kumar Singh (Assistant Professor) and Dr. Khwaja Osama (Professor)** and course coordinator **Dr. Salman Akhtar (Professor)** for their invaluable guidance throughout the course of my dissertation work and academic session. It would have been impossible to complete this work in so short a time without their constant guidance.

I grateful acknowledge **Dr. Dinesh Kumar (Scientist) ,Centre of Biomedical Reasearch , Lucknow** who inspired and encouraged me during my work. I thank him for giving me time in the laboratory and discussing the experiments before performingthem.

I would like to thank respected dissertation coordinators **Dr. Roohi, , Dr. Khwaja Osama** for their continuous support and help.

My acknowledge will be incomplete if I do not mention **my family and friends** with whose support I was able to achieve my goal successfully. There are no words to express my feelings towards them. I silently acknowledge my debt to them.

Priya Agrawal

TABLE OF CONTENT

Abbreviation

ABBREVIATION	FULL FORM
1. µPADs/ mPADs	Microfluidic Paper-based Analytical Device.
2. PADs	Paper-based Analytical Device.
3. POC	Point of Care
4. Approx.	Approximately
5. Etc.	Et cetera
6. WHO	World Health Organization
7. ASSURED	Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, Deliverable
8. ALT	Alanine aminotransferase
9. IUPAC	International Union of Pure and Applied Chemistry
10. UV	Ultra violet
11. TB	Tuberculosis
12. HIV	Human Immunodeficiency Virus
13. ANOVA	Analysis of Variance

List of Figures

List of Tables

List of Equations

1. Introduction

In the last decade, a new generation of analytical devices has emerged based on the cellulose materials – so-called microfluidic paper-based analytical devices (μPADs) – a field that will change the face of the diagnosis of different diseases and sensing of a wide range of biological/chemical/biochemical phenomena. Paper has been used over the centuries for various experimental purposes such as litmus paper as pH indicator, while the first microfluidic paper-based analytical device (μPAD) was introduced by Whitesides and colleagues in 2007.The field of μPADs has continued to develop at an exponential rate with notable impacts on the academic and industrial communities. These devices use cellulose as substrate to serve as paper-based analytical devices (PADs) for the point-of-care diagnosis, bio sensing, environmental monitoring, biomedical and pharmaceutical analysis, clinical diagnosis, and forensic investigations. Furthermore, paper has played an important role in chemical/biochemical analysis, including home pregnancy tests, paper chromatography,paper-based colorimetry, paper-based filtration and purification, pH test, etc. The popularity of PADs is based on several advantages, including very low-cost, power-free due to cellulose fiber networks compatibility with small volume of samples, the ability to store reagents, easy operation and construction, portability and disposability. The μPADs are used in various fields, including environmental monitoring they of contaminations, food safety, health diagnostics, biodefence (micro-organisms sensing), and drug discovery as well as a biomarker and single cell detections. In heath diagnostics it has application in- Analysis of biomolecules such as proteins, hormones, neurotransmitters, etc. Analysis of small molecules such as glucose, uric acid, etc. Nucleic acid analysis. Immunoassays for infectious diseases and cancer detection. Pregnancy tests. Blood typing and blood filtering. Drug sensing. In environmental it has following application in- Water, soil, air analysis, metal detection etc. Food and Beverage control, pesticide, foodborne detection. Water and wine quality analysis etc. The selection of a paper is largely dependent on the application and construction method. In the last years, Whatman® grade 1 filter, which is one of the standard grade filters, has widely been used in the construction of sensors and microfluidics, in large part because of their suitable flow rate, porosity, and particle retention. The wax printing method is a low-cost and simple approach for the construction of the hydrophobic barriers. The μPADs are in early development stage and are going to become very popular and user-

friendly devices in the near future. Taken all, it should be highlighted that the µPADs will change the path of the pharmaceutical research and development and medical sciences in the very near future, opening a new horizon in drug discovery and diagnosis.

Objectives

1. To optimize the process parameters critical to the fabrication process. It discusses the selection of the melting temperature and melting time required to generate impermeable hydrophobic barriers.

2. Determination of the Total Amino Acid Content in blood serum samples using Paper based microfluidics.

2. Literature Review

Paper-based microfluidics is the science and technology of devices made from paper, or other porous membranes, that manipulate small (10^{-6} to 10^{-9} L) volumes of fluids by capillary action. It introduces an innovative platform technology for fluid handling and analysis, with wide range of applications, promoting low cost, ease of fabrication/operation and equipment independence **(H.H Bau *et al.*,2007)**

Paper is defined traditionally as a flexible sheet made from an interlaced network of pressed cellulose fibers. In the context of paper-based microfluidics, paper is defined more broadly as any porous membrane that wicks fluids by capillary action. Paper has been used extensively as a platform for performing analytical assays since the 19th century, beginning with litmus paper**(Rozand *et al.*,2014)**

Two of the most common types of porous membranes used for assays are cellulose-based paper and nitrocellulose membranes. Cellulose-based paper is hydrophilic in nature and is made of cellulose fibers with a high density of hydroxyl functional groups and few carboxylic acid groups Filter paper and chromatography paper are the types of cellulose-based paper used most commonly for the development of microPADs(**W.Shen *et al.*,2011)**

Nitrocellulose is produced by nitrating cellulose (i.e. by replacing hydroxyl groups with nitrate groups). The resulting polymer is then cast into membranes with controlled porosity. The lack of flow control is the main problem of paper-based analytical devices, which generates obstacles for marketing and slows down the transition of paper devices from the laboratory into the consumers' hands. Paper's fibrous and porous structure provides Capillary action- leading to the transportation of the liquids without a need of an external force, absorbency- enabling the storage of the reagents inside the paper, air permeability- removing the air bubble problem, a network structure- enabling the filtration of the sample and a high surface to volume ratio- increasing the number of possible reagents immobilized, causing a considerable fall in the time for the analysis. Immobilization of reagents is achieved due to its natural, high biocompatibility (which has a significant meaning for the samples), biodegradability, disposability and chemical and biological inertness. mPADs have several advantages such as **(S.S Sibbett *et al.*,2008)**

(1) it is a ubiquitous and extremely cheap cellulosic material; (2) it is compatible with many chemical/biochemical/medical applications; and (3) it transports liquids using capillary forces without the assistance of external forces. By building microfluidic channels on paper, liquid flow is confined within the channels, and therefore, liquid flow can be guided in a controlled manner. A variety of 2D and even 3D microfluidic channels have been created on paper, which are able to transport liquids in the predesigned pathways on paper. At the current stage of its development, paper-based microfluidic system is claimed to be low-cost, easy-to-use, disposable, and equipment-free, and therefore, is a rising technology particularly relevant to improving the healthcare and disease screening in the developing world. However, it also has disadvantages as a material for diagnostic devices: lack of flow control, it is also not well suited for absorbance measurements because the paper fibers lead to large amounts of light scattering, the wicking rate of paper is not always perfectly uniform even within a single sheet of paper.

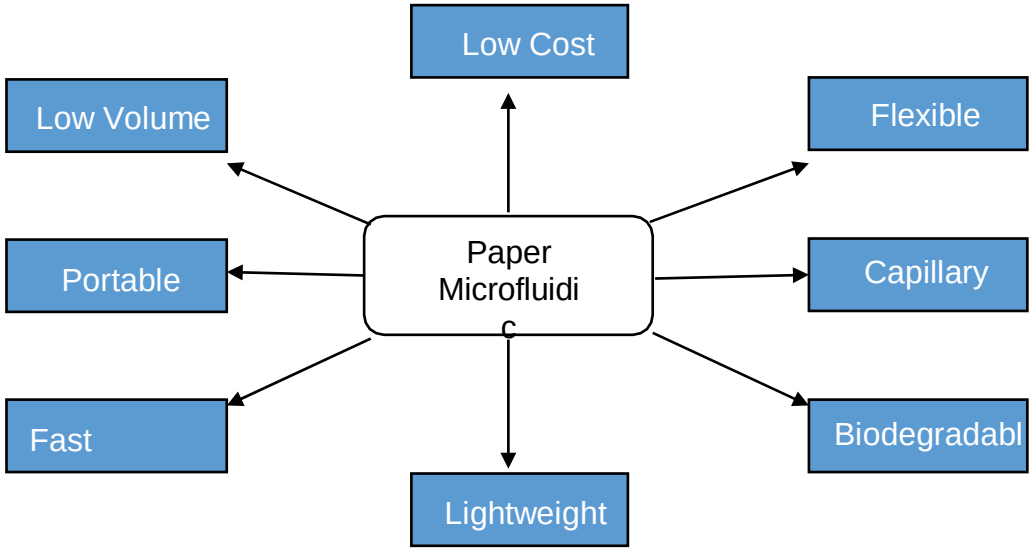
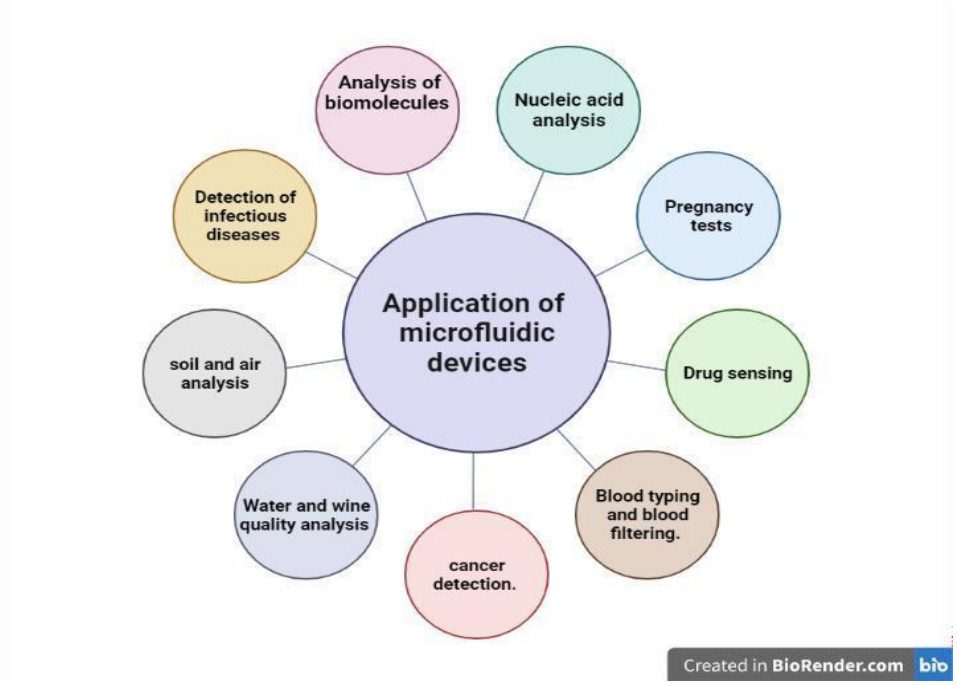


Figure 1. Key points of microfluidic paper-based analytical devices (μPADs)

2.1 Application:

The main application of paper-based microfluidic devices is to provide a low-cost, easy-to use, and portable analytical platform for assays, either multi-analyte or semi-quantitative (even quantitative), in order to provide people living in the developing world with affordable disease diagnosis and environmental monitoring. μPADs are of critical importance since they enable POC detection and can ideally be used massively in low income countries, where people predominantly die of preventable or curable infectious diseases: lower respiratory infections, HIV/AIDS, diarrheal diseases, malaria and tuberculosis collectively, accounting for almost one third of all deaths in these countries. While significant progresses have been made in interventions to prevent and treat most of these diseases, often the effort are not widely reaching all population due to the lack of laboratory infrastructure, trained personnel and financial support. Therefore, affordable, equipment free, simple to operate, and robust diagnostic assays at the point of care would be considered as life savers under these resource-limited conditions. The aim of μPADs for POC is to obtain the results quicker in order to take clinical decisions instantly and to carry out the treatment plan immediately. Moreover, μPADs fulfil the World Health Organisation (WHO) ASSURED criteria for an ideal rapid test: Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment free, Deliverable to end users, delivering affordable POC diagnostic tests.



Additionally, early diagnosis of diseases like cancer, where the detection and sometimes quantification of molecules and biomolecules like glucose, lactate, uric acid, alcohol, biomarkers for liver function, ATP, ALT, nitrite, nucleic acids among others is needed in physiological fluids like blood and urine, can be successfully accomplished by μPADs. The potential of μPADs is not only limited to healthcare; devices developed for environmental safety, detecting water, soil or air contamination, and for food and beverage control are also available at the laboratory state. In the resource-limited countries or after a natural disaster like an earthquake, to be able to determine drinking water quality with an easy to use and low cost paper-based device would be very beneficial. Furthermore, the possibility of controlling the quality of foods and beverages by the final consumer and ensuring the control over the whole food/beverage production chain would be an important advantage for farmers, production companies and sellers in order to generate a more dynamic market. Therefore, it is here where μPADs are realistic alternatives for low cost, mass production and marketable devices.

2.1.1 Colorimetric Test:

Colorimetric analysis refers to a quantitative technique used to measure the concentration of a given substance in a solution. This allows the quantification of substances such as water and chemicals on metallic surfaces and their corresponding contribution to corrosion rates. This test process is particularly useful since most test substances are not readily discernible by the human eye. Colorimetric analysis is a simple mechanism to make test substances identifiable.

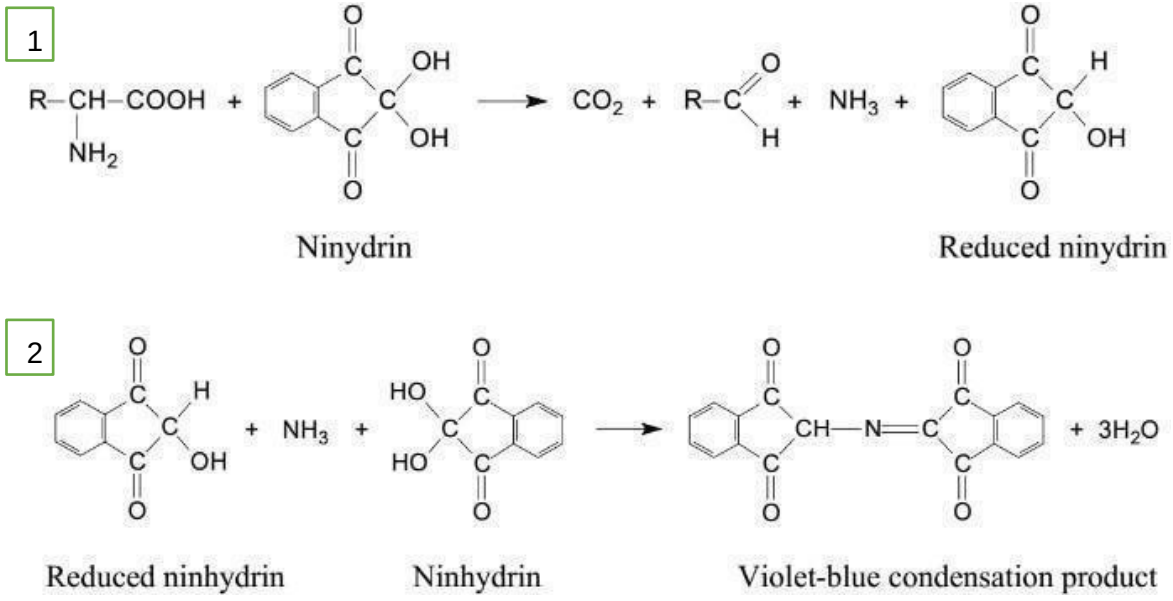
The ninhydrin test is a chemical test which is used to check whether a given analyte contains amines or α-amino acids. In this test, ninhydrin (a chemical compound with the formula $C_9H_6O_4$; IUPAC name: 2,2-dihydroxyindane-1,3-dione) is added to a test solution of the analyte. The development of a deep blue colour indicates the presence of ammonia, primary/secondary amines, or amino acids in theanalyte.

Principle: The amino group belonging to a free amino acid undergoes a chemical reaction with ninhydrin, which behaves as an oxidizing agent. When exposed to ninhydrin, the amino acid undergoes oxidative deamination, resulting in the liberation of

CO_2, NH_3, and an aldehyde along with hydrindantin (which is a reduced form of ninhydrin) as shown in reaction 1 below.

Now, the ammonia goes on to react with another ninhydrin molecule to form diketohydrin (which is also known as Ruhemann's complex). This complex is responsible for the deep blue colour as shown in reaction 2 below. When the analyte contains Imino-acids like proline, a yellow coloured complex is formed. When asparagine is used, the colour of the resulting complex is brown.

Reaction involved:



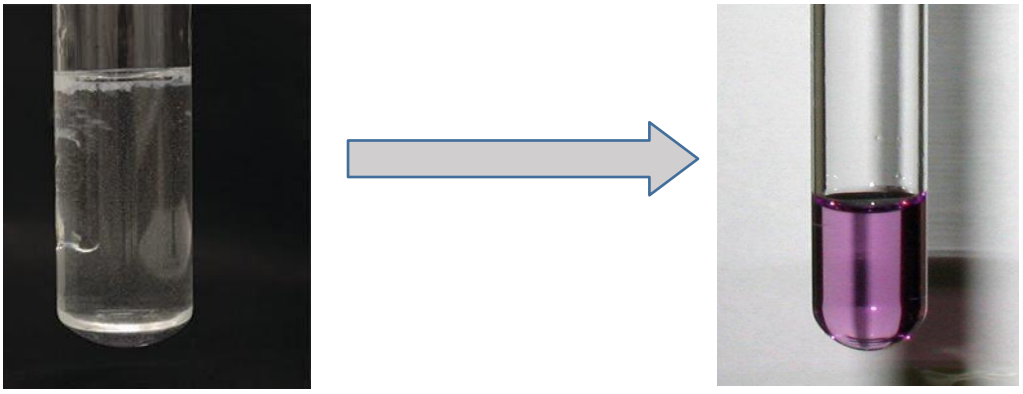


Figure 3. Glutamic acid produces violet-blue colour complex when tested with ninhydrin reagent.

2.1.2 Fabrication:

The objective of every technique for patterning paper is to create well-defined patterns of hydrophobic barriers in a piece of paper to define hydrophilic channels and zones.

It is a simple and inexpensive method for fabricating microfluidic devices in paper using a bronze stamp and hot plate. The stamp is heated on the hot plate upto an optimized temperature then it is stamped on whatman filter paper beneath which a wax coated whatman filter paper is placed. This process creates complete hydrophobic barriers in paper that define hydrophilic channels, fluid reservoirs, and reaction zones.Hydrophobic agents are directed to the selected areas of a sheet of paper to hydrophobize these areas; areas not receiving patterning agents remain hydrophilic. Wax is a cheap hydrophobic material, which has been widely used in the fabrication of mPADs. Due to the porous structure of the Filter paper, the wax penetrates into the paper to form well-defined micro-channels on the paper. These hydrophobic agents change the wetting property of paper by filling the paper pores or adsorb on the fiber surface.

A variety of hydrophobic substances have been utilized to define hydrophilic micro-channels on paper, from the relatively expensive agents such as photoresist SU-8 ($0.1 for patterning filter paper of 100 cm2), 20 the less expensive agents such as wax ($0.01 for patterning filter paper of 100 cm2) 13 to the extremely cheap agents such as alkyl ketene dimer (AKD, $0.00001 for patterning filter paper of 100 cm2).

Based on the binding states of hydrophobic agents to paper, the paper patterning principles of these techniques can be divided into three categories: physical blocking of the pores in paper (using agents such as photoresist and polydimethylsiloxane (PDMS)), physical deposition of a hydrophobizing reagent (e.g., polystyrene or wax) Physical pore blocking and physical fibre surface modification do not involve any chemical reactions between the hydrophobic agents and the cellulose fibres; these agents are physically impregnated in paper pores or deposited onto the fibre surface. The presence of these agents changes the liquid wetting properties of the paper, making the formation of hydrophilic-hydrophobic patterns in paper possible.

The patterns were printed on Whatman no. 1 chromatography paper using the the bronze stamp. The bronze can print an 8.5 in. × 11 in. sheet of paper in approximately 45 s. The

wax melted and spread through the thickness of the paper. The patterned paper was ready for use and allowing it to cool to room temperature (<10 s). Another economical stamping technique was also introduced where the design of the microfluidic structure was patterned in a lightweight and portable stainless steel stamp for rapid prototyping of mPADs, using paraffin over a chemically modified paper substrate. Preheating of the stamp and oxidisation of the paper surface were additional steps that could be considered drawbacks. In general, stamping methods are ideal for easy and portable fabrication of mPADs, but they are considered weak methods for mass production. Nevertheless, it needs to be considered that they are good device candidates to be used in field and under resource limited settings.

There are various types of fabrication methods, some are explained below;

2.1.2.1 Paper microfluidic device: Wax Printing

It is adapted to fabricate large numbers of paper analytical devices in a single batch due to its rapidity (5-10 min) and low cost. The two main steps in the fabrication process are printing patterns of wax (100 μm width) on the paper surface and melting the wax into the paper to form hydrophobic barriers. There are few different ways to realize wax patterning, and direct printing by a wax printer is the most convenient and efficient method. As an alternative, painting with a wax pen is also possible to perform, though some prefer to print patterns with a normal inkjet printer, and then trace and paint with a wax pen for a better resolution of the printed pattern.

2.1.2.2 Paper microfluidic device: Inject Printing

Inkjet printing is a new fabrication method that associates sizing chemistry with digital inkjet printing technique. The main objective here in the fabrication of microfluidic channels is to obtain a contrast between the hydrophobic barrier and the hydrophilic flow channel. Inkjet printing systems employed to print patterns rely on drop-on-demand technology (DOD), which enable the jetting of ink droplets dot-by-dot onto cellulose paper only when it is needed. This make inkjet printing a technique that allows for rapid and flexible high resolution. The Inkjet printing can accurately deliver biomolecules and indicator reagents into the microfluidic patterns, thus forming biological/chemical sensing

zones within the patterns. To define channels on the paper substrate, the cellulose fibers can be covalently modified by employing sizing agents such as alkenylsuccinic anhydride (ASA), alkyl ketene dimer (AKD), or rosin. Those hydrophobic reagents render the paper substrate more hydrophobic, and AKD has been particularly used for the microfluidic patterning of paper by inkjet printing.

2.1.2.3 Paper microfluidic device: Photolithography

This method involves light exposure through a mask to project the image of a pattern, much like a negative image in standard photography. Photolithography is a convenient, quick, and cheap method, and with this technique, hydrophobic areas that compose the patterns are made of polymeric barriers. Channels created using Photolithography show high background while wax printed channels for instance show very low background. However, photolithography requires organic solvents, expensive photoresists and photolithography equipment, making this method a little more complicated to set up. Fast Lithographic Activation of Sheets, a variation technique based on photolithography is a rapid method for laboratory prototyping of microfluidic devices on paper. It requires a UV lamp and hotplates only and patterning can even be performed in sunlight when the UV lamp and hotplate are unavailable and no clean room or special facilities arerequired.

2.1.2.4 Paper microfluidic device: Flexographing Printing

This method for patterning is based on flexographic printing of polystyrene, a polymer used to make the paper substrate hydrophobic. This technique leads to the formation of liquid guiding boundaries and layers on paper substrates. As a result, hydrophobic barrier structures are created, and the hydrophobizing inks partially or completely penetrate through the entire depth of the paper substrate. Thus, the structures obtained are very thin fluidic channels on paper with reduced sample volumes. A great advantage of flexographic printing is that biomolecules and other reagents required in analytical and diagnostic tests can easily be transferred by it on paper substrates. The fabrication of paper microfluidic analytical devices using flexographic printing can be done in a single roll-to-roll process, and that’s the reason why this method is ideal for largescale production.

2.1.2.5 Paper microfluidic device: Wax screen-printing

Wax screen-printing is a low-cost and simple method for fabricating paper microfluidic analytical devices. It's simple fabricating process includes printing patterns of solid wax on the surface of paper using a simple screen-printing method. The printed wax is then melted into the paper to form hydrophobic barriers using a hot plate. As previously seen, wax is a low-cost material and can be purchased anywhere in the world, and is also environmentally friendly. This method requires a wax printer and easily affordable printing screens. Besides, the wax screen-printing method is accomplished without the use of clean room, UV lamp, organic solvents, or complexed instrumentation. Another major advantage of this method over previous methods is that it requires only a common hot plate (or similar surface) and common printing screen that can be produced anywhere in the world, making it ideal for fabrication of the μPADs in developing countries. Finally, this fabrication method is useful for both colorimetric and electrochemical detection methods.

2.1.2.6 Future prospect:

Paper diagnostics are manufactured with the help of cellulosic materials as well as paper in order to quantify and identify chemical agents as well as biomolecules which are hazardous to the health. The demand for paper diagnostics is increasing due to several properties such as easy to function, hydrophilic, sterilisable, biocompatible, combustible, biodegradable, easily engineered, widely available and cheap. The requirement for cost-effective solutions results in generating demand for paper diagnostics. Also, a rise in the usage of pregnancy test kits and diabetes test kits are driving the growth of the market. Moreover, factors such as rise in the obesity, unhealthy diet, smoking and other lifestyle habits are expected to generate higher growth of the market. It is also due to rise in the incidence of diseases such as cardiac diseases and diabetes. The product segment includes paper based microfluidics, dipsticks and lateral flow assays. Lateral flow assays has the highest growth in the paper diagnostics market. It is due to increased usage of pregnancy test kits as well as its applications in various products. Also, rise in

the infectious diseases like pneumonia, TB, HIV are expected to boost the growth of the market over the forecast period. Increased investments and efforts by the healthcare organizations and the government in order to treat as well as detect TB are driving the growth of the market.

The devices type segment includes monitoring devices and diagnostic devices. Diagnostic devices have the largest market share in the paper diagnostic market. Such devices are primarily utilized in glucose detection and blood separation. Contributing factors to the growth of the market are new product launch, frequency of diagnosis, quality and increased awareness regarding the devices. Wax patterning technology has higher adaptability and flexibility which result in increasing the demand for diagnostics devices. Also, increased demand for urinalysis is generating higher demand for the market.

The market has been divided into North America, Europe, Asia-Pacific, Middle East & Africa, and South America. North America has the highest growth due to a strong technological distribution network along with presence of major headquarters of the key players. Increased initiatives by the government regarding advanced diagnosis is contributing to the growth of the market in the region. Some of the notable players in the global paper diagnostics market are Acon Laboratories Inc., Bio-Rad Laboratories, ARKRAY Inc., Siemens healthcare Gmbh and Abbott.

Although new fabrication methods of paper-based microfluidic devices will continually be reported in future, the practicality of the existing and future methods will be judged by the POC (point of care) and diagnostic market in terms of the material and fabrication costs, their potentials for mass productions, their reliance upon any other equipment in order to function, their reliability in providing easy-to-interpret assay results, and their compatibility to telemedicine, particularly with mobile phone transmission or interpretation of test results.

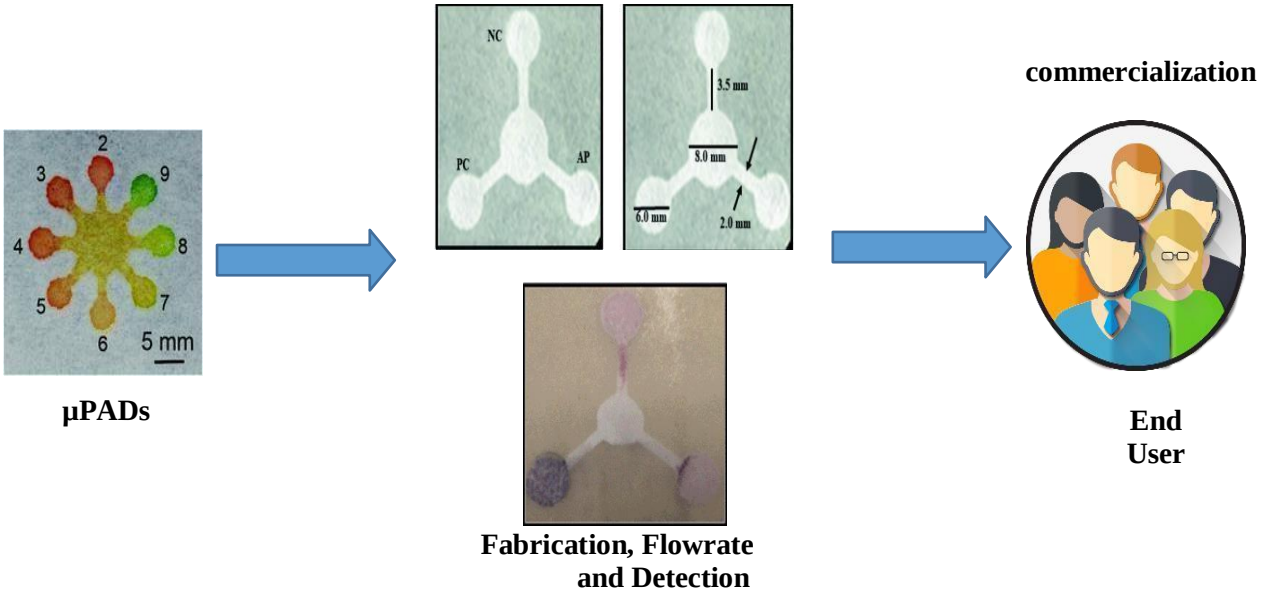


Figure 4. Commercialization of µPADs.

3. Material and Method

3.1 Fabrication of Microfluidic Device:

The fundamental principle underlying these fabrication techniques is to pattern hydrophilic hydrophobic contrast on a sheet of paper in order to create micron-scale (i.e., hundreds to thousands of micrometres) capillary channels on paper. To choose the proper technique, researchers and manufacturers should consider a range of factors including equipment availability, material costs, fabrication process simplicity, and the intended applications of paper-based microfluidic devices. At present, AKD ink jet printing and wax printing might be the most promising techniques due to the low cost of patterning agents and easy, rapid fabrication process.

Steps involved in fabrication of device:

1. Wax sheet preparation: A piece of filter paper was immersed in liquid paraffin and solidified at room temperature. This Wax sheet (p-paper) was then placed on the native paper (n-paper) surface.
2. Stamping: A metal stamp was preheated and brought in contact with the paraffin Coated paper to stamp the microfluidic structure on the n-paper, which transferred the paraffin from the p-paper to the n-paper and formed the hydrophobic barriers. As shown in figure 5 below.

3.2 Wax sheet preparation:

The Whatman filter paper was dipped into paraffin wax (melting point > 65°C), after it was set dry another fresh Whatman paper was placed upon the waxed coated sheet and it is then pressed by the heated stamp to create a hydrophobic barrier. The area of the paper penetrated by the wax is hydrophobic, whereas the paper itself is hydrophilic. Functionalized paper based microfluidic device were dried at room temperature and used the same day.

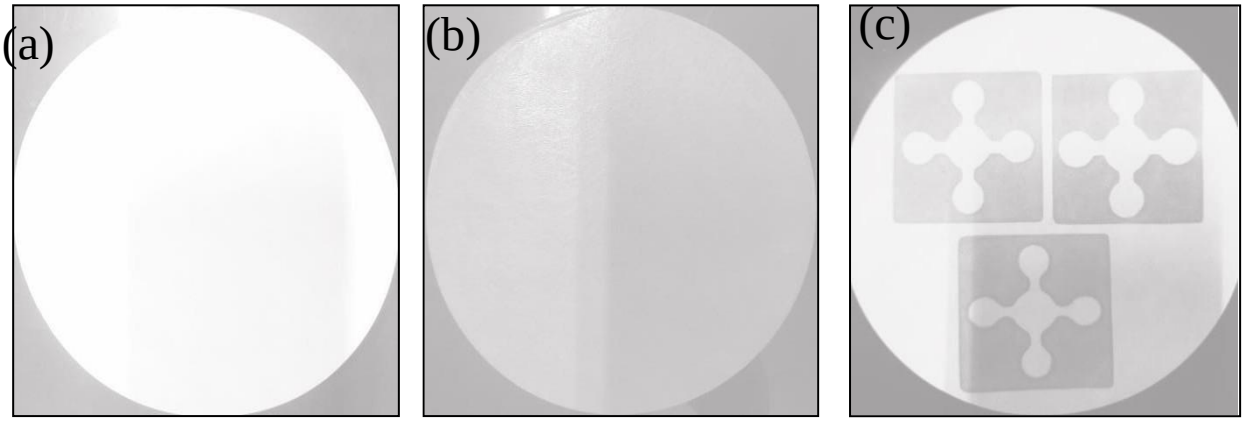


Figure 6. **(a)** shows Whatman filter paper (n-paper), **(b)** shows coated Whatman filter paper (p-paper), **(c)** fabricated sheet having reservoirs obtained by stamping.

3.3 Stamping:

The objective of every technique for patterning paper is to create well-defined patterns of hydrophobic barriers in a piece of paper to define hydrophilic channels and zones. A microfluidic paper-based analytical device (μPAD) was fabricated by stamping the hydrophobic barrier pattern onto a laboratory filter paper. The μPAD has a central zone from which an applied sample flows into four surrounding narrow channels to which had been added the standard solutions. Each channel is connected to a circular area loaded with the reagent.We demonstrated that, by taking advantage of the absorbing capability of filter paper, it is possible to create paper-based microfluidics by simple contact stamping. Pressure is required for thermal transfer of paraffin onto surface of filter paper. The design of the microfluidic structure has been patterned in a stamp, machined in stainless steel. A filter paper sheet was impregnated with paraffin and sandwiched with a native paper (n-paper) sheet. Using a hot plate (Fig-7B) The metal stamp was preheated at 75°C and then brought in contact with the paraffined paper (p-paper) to enable the thermal transfer of the paraffin to the n-paper, thus forming the hydrophobic barriers by applying pressure for 45 s. As explained in Figure 18.

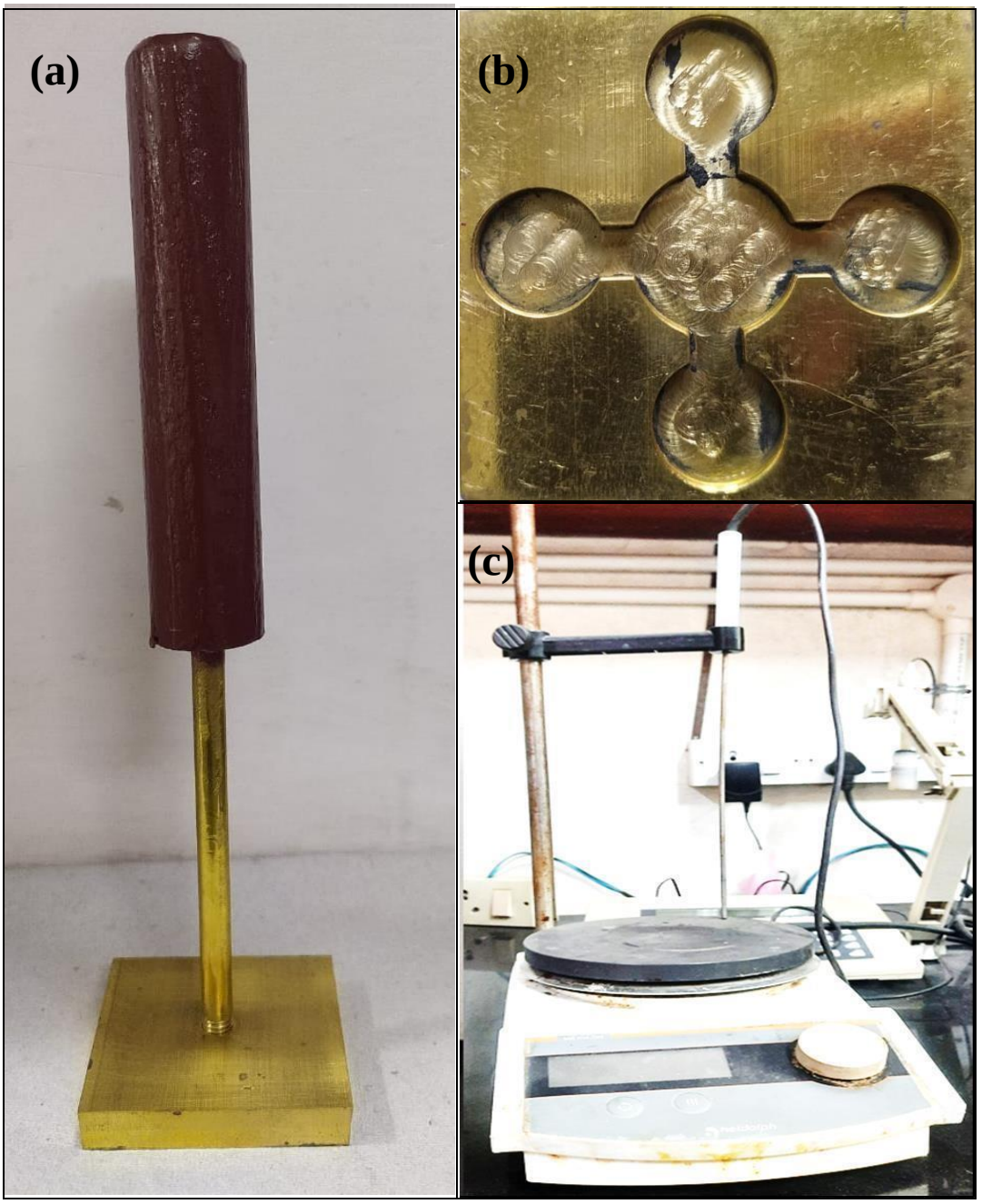


Figure 5.(a) Bronze Stamp, **(b)** Engraved channels on bronze stamp.
(c) Hotplate

3.4 Serum Sample Preparation-

Figure 6. Illustration of serum sample preparation.

Collected blood in a micro centrifuge tube taken out of different patients. After collection of the blood, the blood was allowed to clot by leaving it undisturbed at room temperature. This usually takes 15-30 minutes. Removed the clot by centrifuging at 1,000-2,000 rpm for 10 minutes in centrifuge. The resulting supernatant is designated serum.

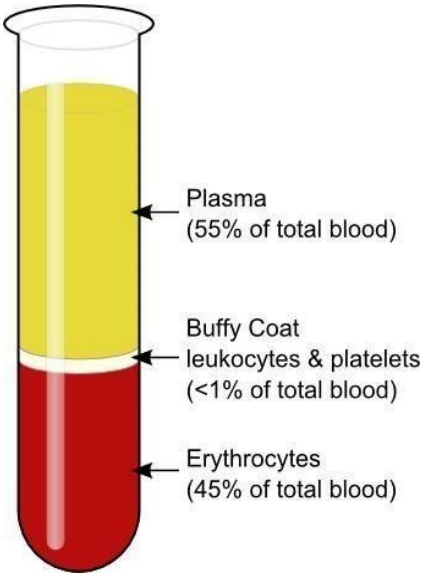
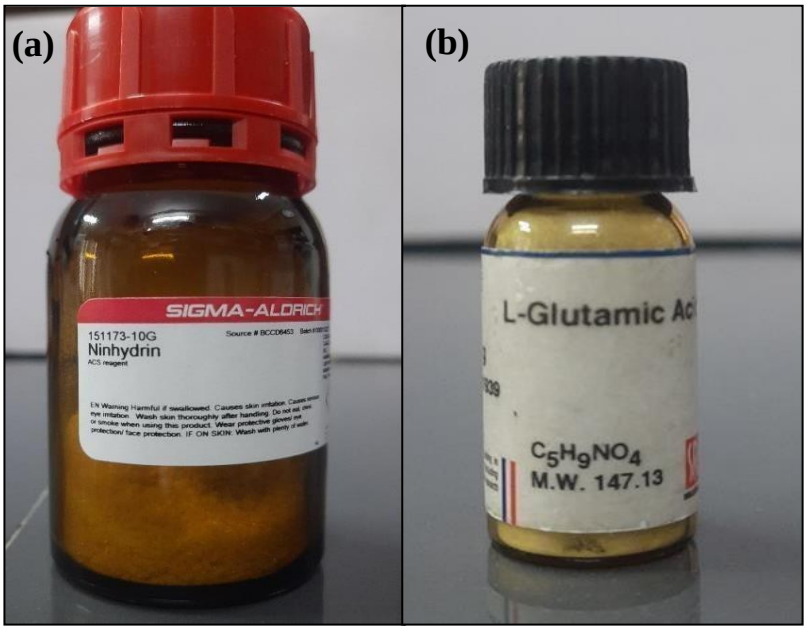


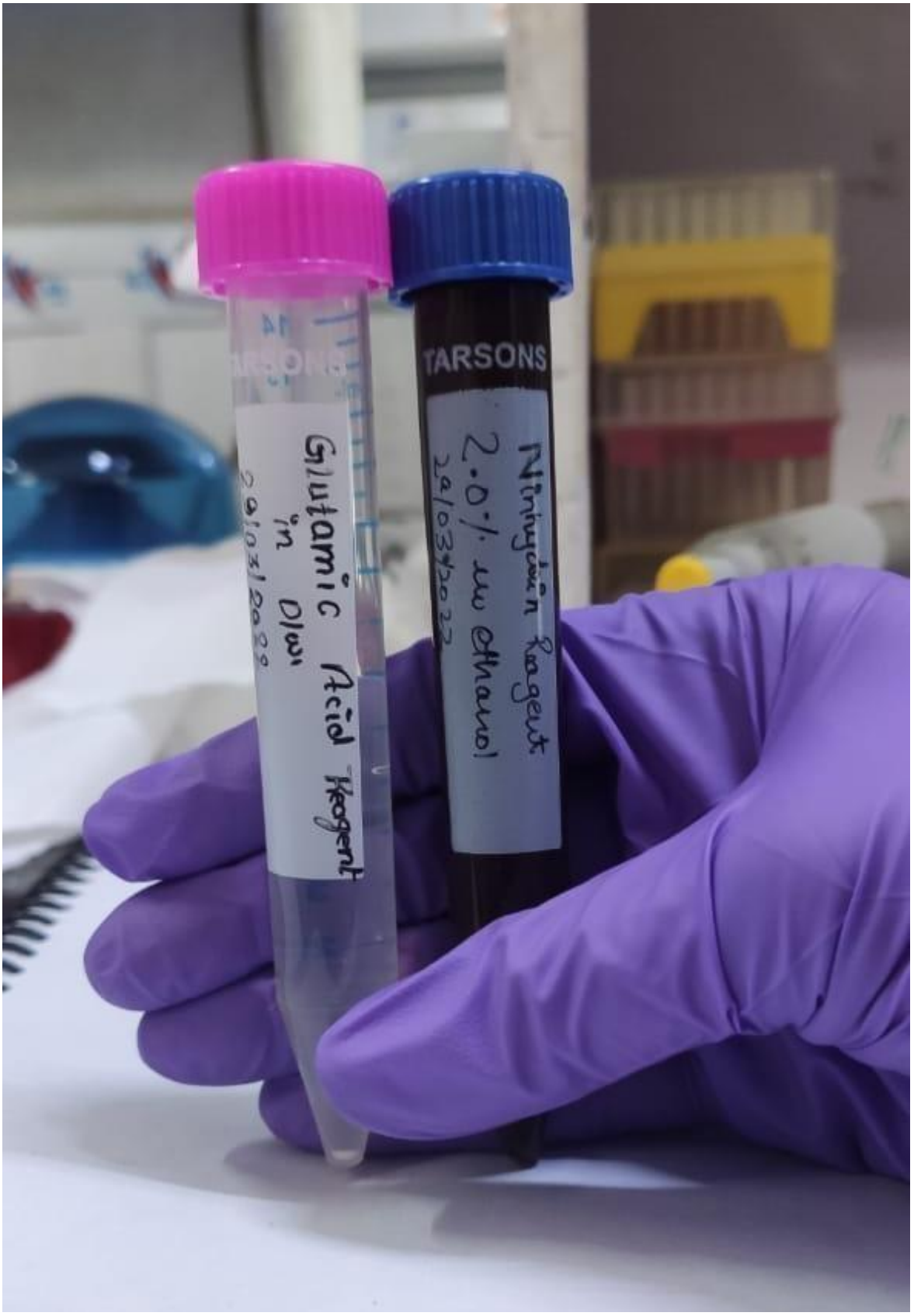
Figure 7. Components of blood sample separated after centrifugation.

3.5 Determination of amino acid:

Material-

All reagents were of analytical grade and distilled water was used. The 2.0% ninhydrin solution was prepared by mixing 200 g of ninhydrin in 10 mL of distilled water. Glutamic acid was used as the reagent for preparing the standard solution of total amino acids.A glutamic acid stock standard solution of 1.0 mg/mL was prepared by dissolving 10 mg of glutamic acid with distilled water and diluted to 10 mL. The glutamic acid working standard solutions were prepared by appropriate dilution of the stock solution with distilled water.





Glutamic acid and ninhydrin reagent

Procedure-

Steps were followed to determine amino acid using ninhydrin test

1. Place a beaker of approx. 40 mL capacity on the experimental table, and then put the dry fabricated paper-based device on the top of thebeaker.

2. Spotted 70 µL of 2.0% ninhydrin on the circle zone in the center of the dry paper-based device. The ninhydrin solution flows into the detection zones within approx. 100 seconds.

3. Allow the device to dry (approx. 9 minutes).

4. Spot 10 µL of each 16 standard solutions glutamic acid (250, 500, 750, 1000, 1250, 1500, 1750, 2000, 2250, 2500, 2750, 3000, 3250, 3500, 3750 µg/mL). Let the solutions dry for 5 minutes at room temperature.

5. Heated the device on a hot plate at 80°C for 15 minutes such that the amino acid reacts with the ninhydrin to forma purple-coloured complex in the detection zones.

6. Taken an image of the device covering all detection zones using a scanner.

7. Analysed the test zones for the mean grey value using ImageJ software.

8. Obtained a calibration curve. The final mean grey values of standards used while creating the calibration curve are obtained by subtracting the grey value of blank.

9. Obtained linear equation for the correlation.

10. Calculated the total concentration of amino acids in sample solution according to the mean grey value in the sample detection zone and the linear equation.

4. Software Used for Analysis-

4.1 ImageJ:

ImageJ is free public domain image processing software developed at the National Institutes of Health. Its power and flexibility allow it to be used as a research tool by scientists in many disciplines, from medicine to astronomy. It can be used to display, annotate, edit, calibrate, measure, analyse, process, print, and save raster (row and column) image data. It reads most common raster image formats as well as raw data files in text format, such as from spreadsheets.

Steps involved in analysis using ImageJ:

Step 1- Image was uploaded as shown in figure 11.

Step 2- Region of interest (ROI) was selected using suitable shape to select the area as shown in figure 12.

Step 3- Further selected the option “Analyse” then in analyse chose the option “measure” which gave results including the area of selected region along with its mean as shown in figure 13.

Step 4- Saved the result and opened it in spreadsheet.

Step 5- Mean grey value was calculated by subtracting the value of blank from all other calculated values.

Step 6- The graph was plotted using scattered graph type and obtained a straight line graph by joining the dots.

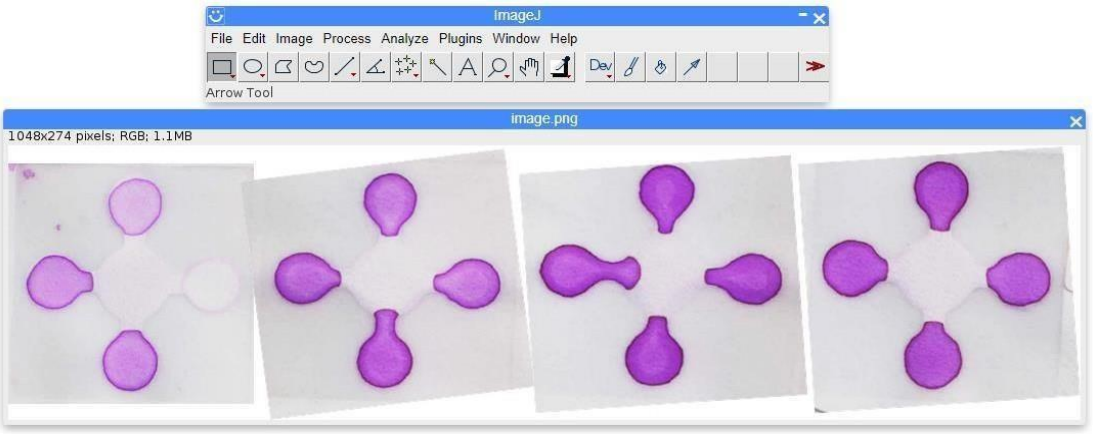


Figure 9. Upload the images.

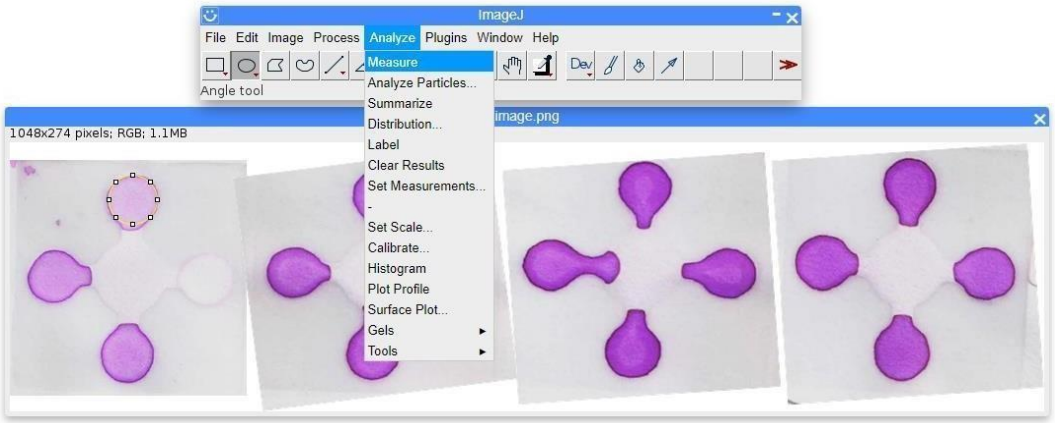


Figure 10. Selection of region of interest. While selecting the region the area must remain same of all other reservoirs.

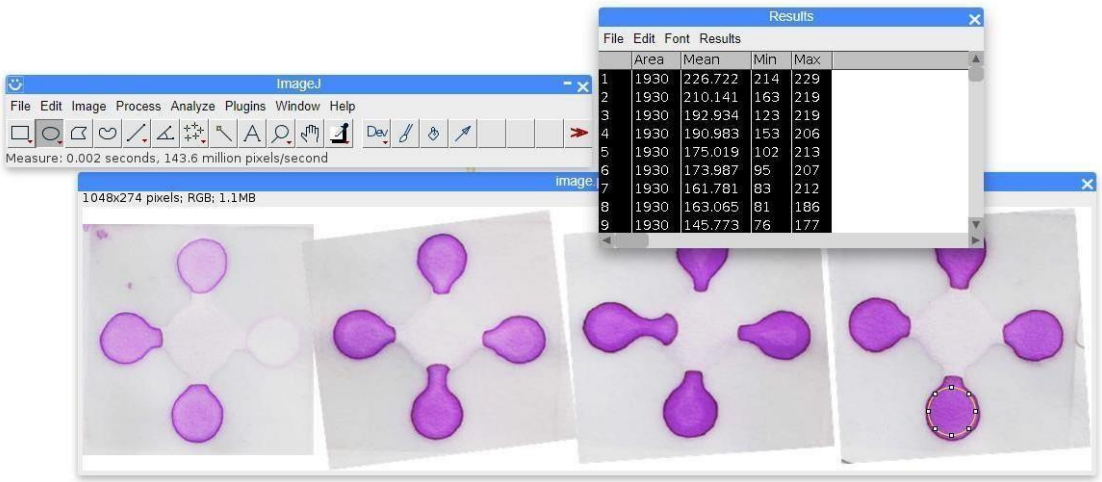


Figure 11. Obtaining the computed result providing mean values for all samples.

4.2 GraphPad Prism:

GraphPad Prism combines scientific graphing, comprehensive curve fitting (nonlinear regression), understandable statistics, and data organization. While it won't replace a heavy-duty statistics program, Prism lets you easily perform basic statistical tests commonly used by laboratory and clinical researchers. Prism offers t tests, nonparametric comparisons, one-, two- and three-way ANOVA, analysis of contingency tables, and survival analysis. Analysis choices are presented in clear language that avoids unnecessary statistical jargon.

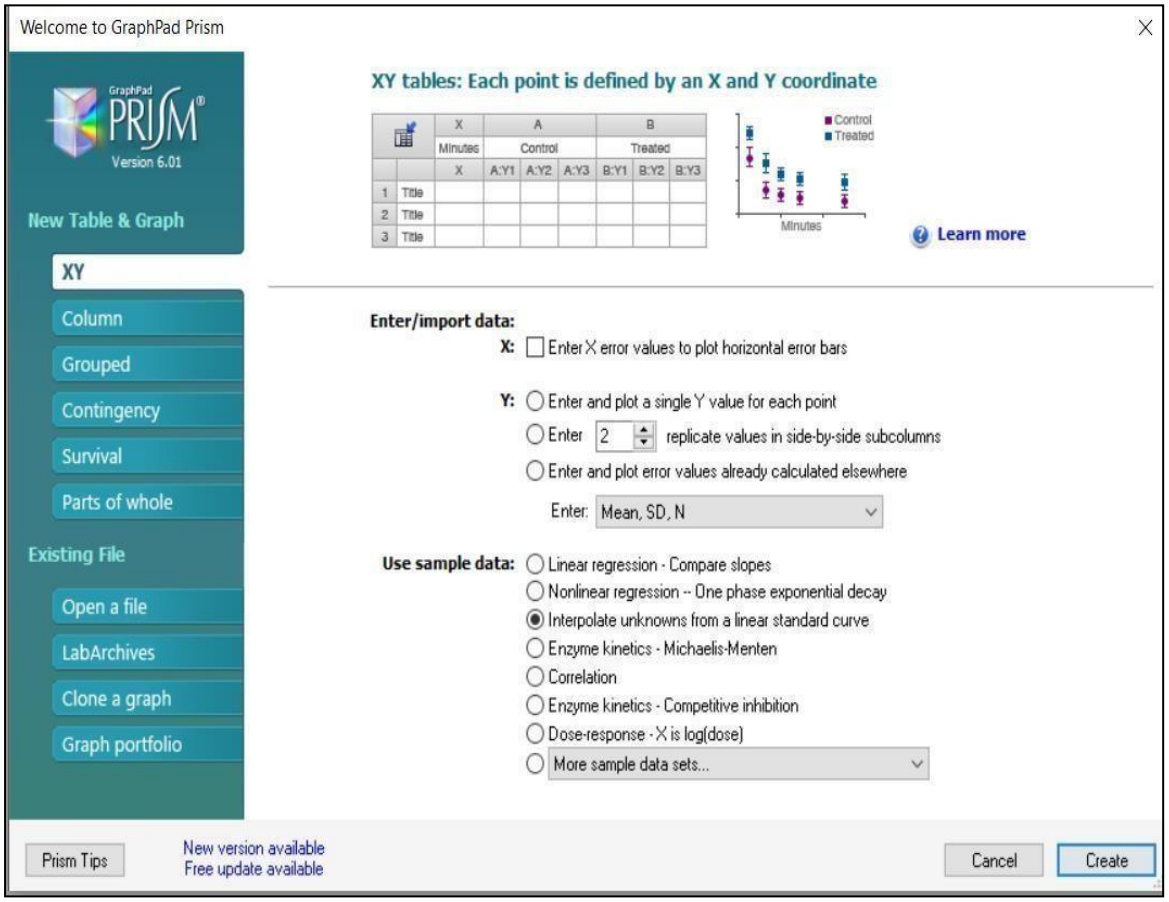
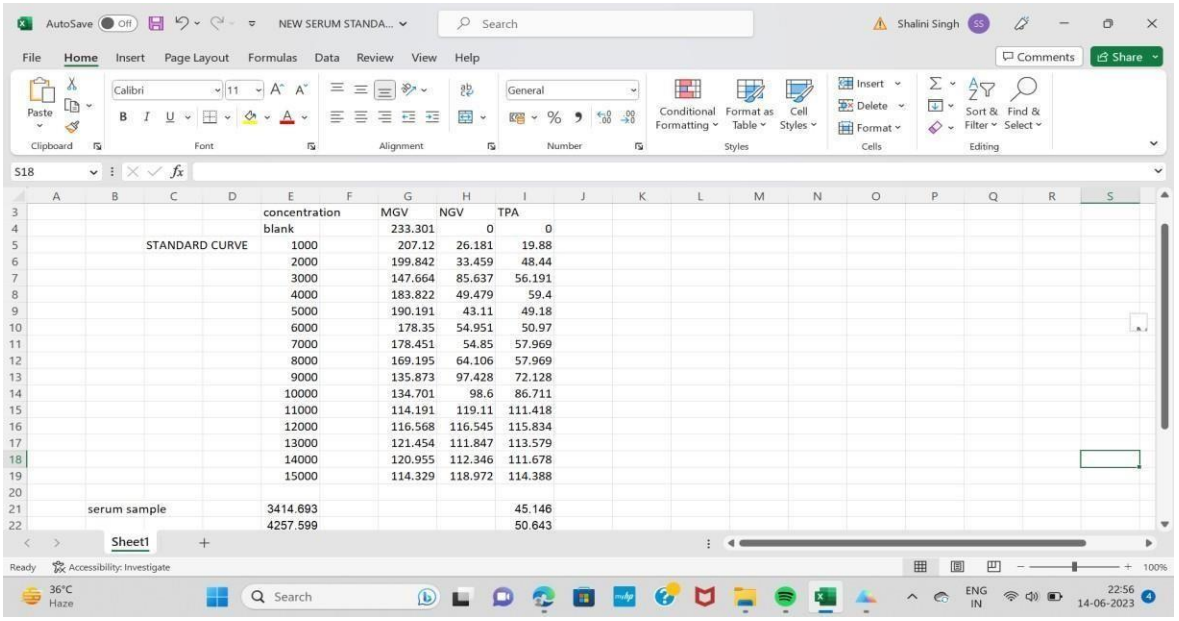
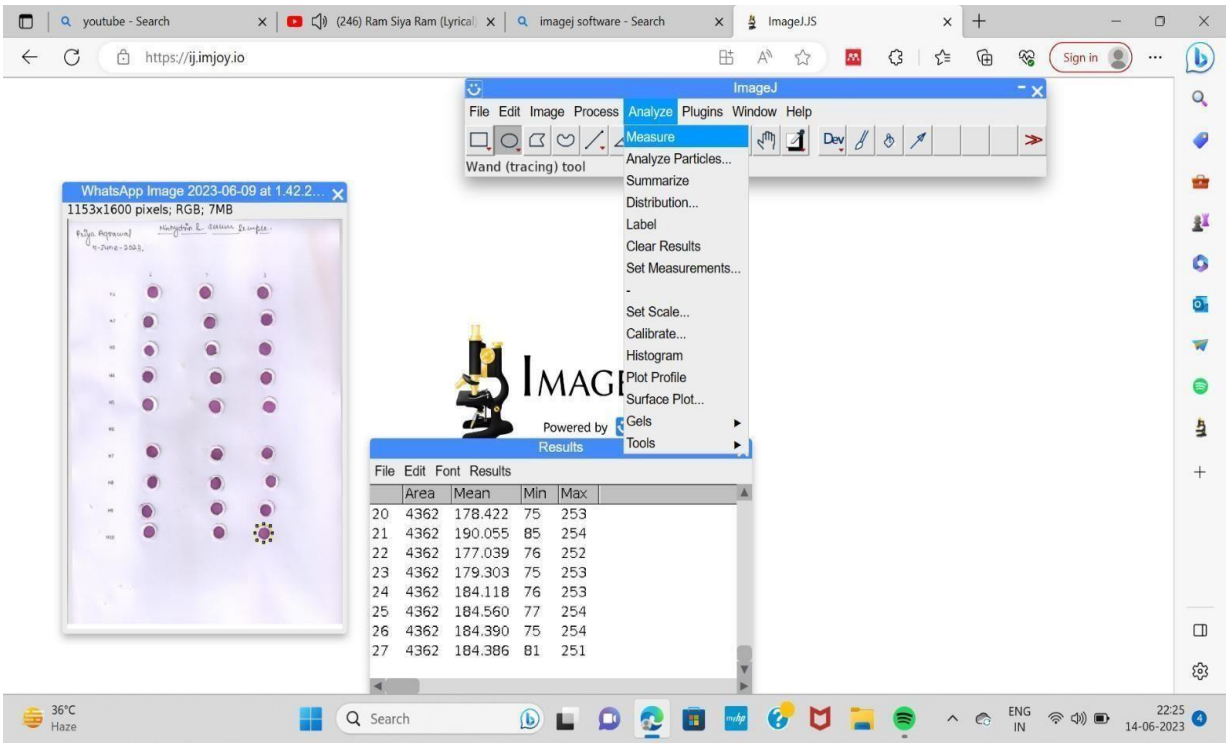


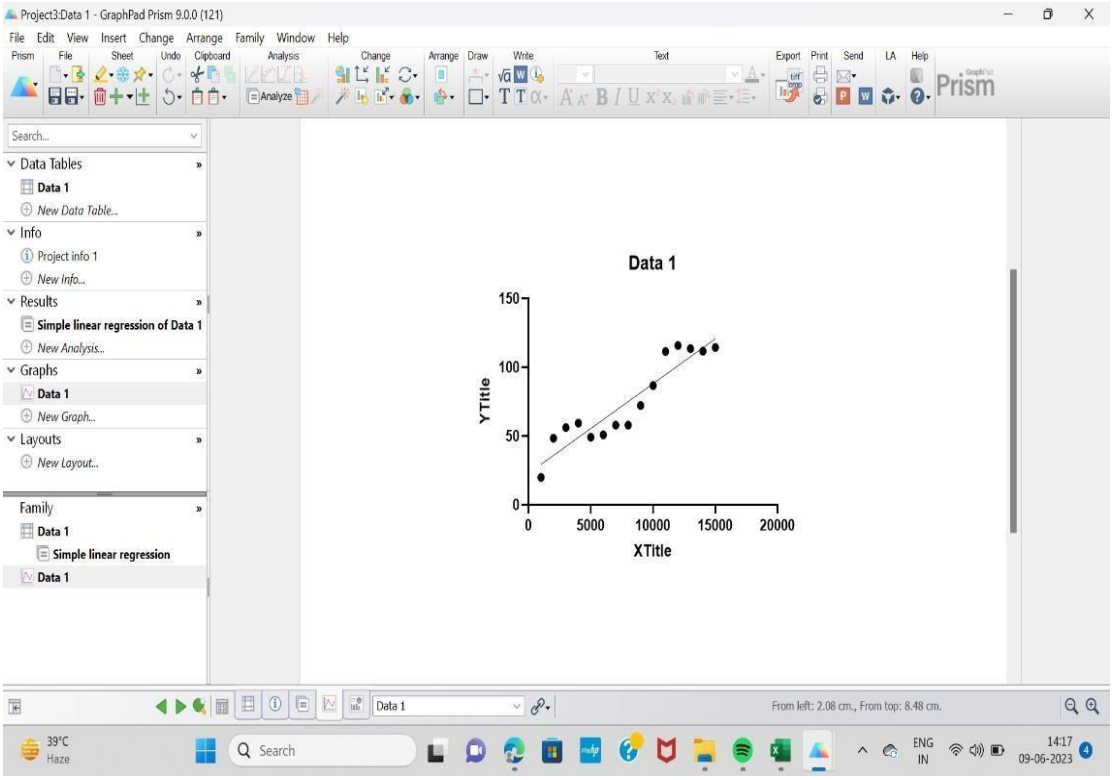
Figure 12 Cover page of GraphPad Prism.

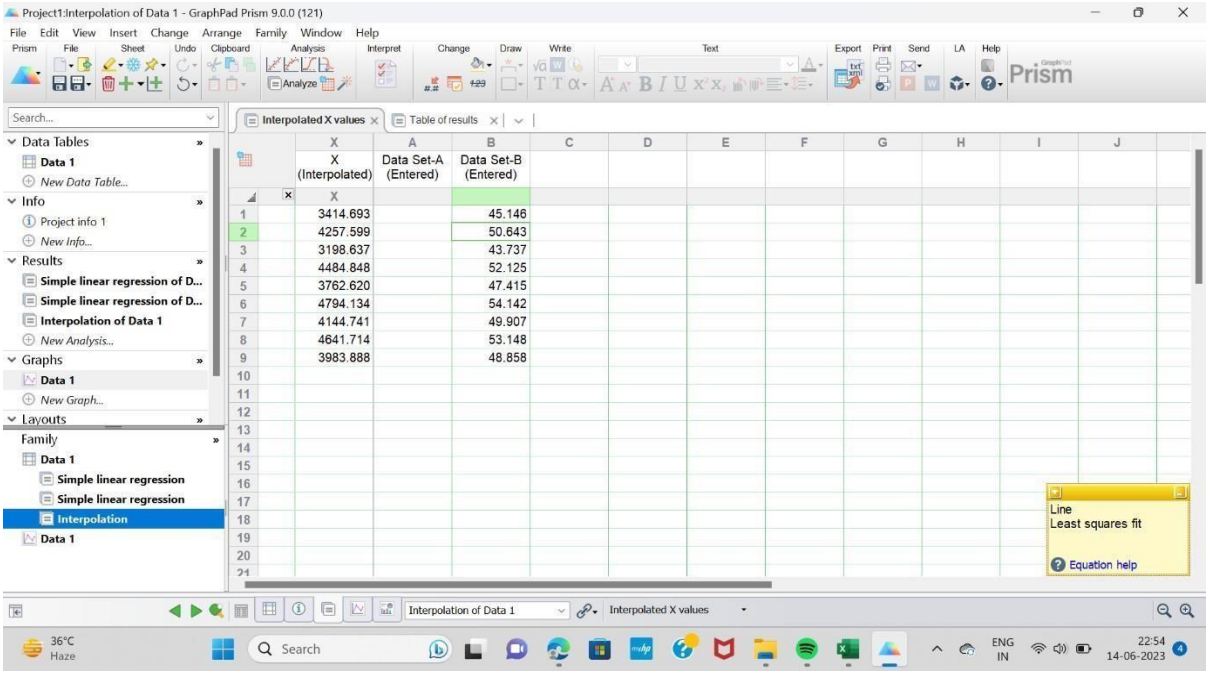


	concentration	MGV	NGV	TPA
blank		233.301	0	0
STANDARD CURVE	1000	207.12	26.181	19.88
	2000	199.842	33.459	48.44
	3000	147.664	85.637	56.191
	4000	183.822	49.479	59.4
	5000	190.191	43.11	49.18
	6000	178.35	54.951	50.97
	7000	178.451	54.85	57.969
	8000	169.195	64.106	57.969
	9000	135.873	97.428	72.128
	10000	134.701	98.6	86.711
	11000	114.191	119.11	111.418
	12000	116.568	116.545	115.834
	13000	121.454	111.847	113.579
	14000	120.955	112.346	111.678
	15000	114.329	118.972	114.388
serum sample	3414.693			45.146
	4257.599			50.643



	Area	Mean	Min	Max
20	4362	178.422	75	253
21	4362	190.055	85	254
22	4362	177.039	76	252
23	4362	179.303	75	253
24	4362	184.118	76	253
25	4362	184.560	77	254
26	4362	184.390	75	254
27	4362	184.386	81	251


Project3:Data 1 - GraphPad Prism 9.0.0 (121)
File Edit View Insert Change Arrange Family Window Help
Data Tables
Data 1
New Data Table...
Info
Project info 1
New Info...
Results
Simple linear regression of Data 1
New Analysis...
Graphs
Data 1
New Graph...
Layouts
New Layout...
Family
Data 1
Simple linear regression
Data 1
Data 1
150
100
50
0
YTitle
0
5000
10000
15000
20000
XTitle
From left: 2.08 cm., From top: 8.48 cm.
39°C
Haze
Search
ENG
IN
14:17
09-06-2023


Project1:Interpolation of Data 1 - GraphPad Prism 9.0.0 (121)
File Edit View Insert Change Arrange Family Window Help
Interpolated X values
Table of results
Data Tables
Data 1
New Data Table...
Info
Project info 1
New Info...
Results
Simple linear regression of D...
Simple linear regression of D...
Interpolation of Data 1
New Analysis...
Graphs
Data 1
New Graph...
Layouts
Family
Data 1
Simple linear regression
Simple linear regression
Interpolation
Data 1
X (Interpolated)
Data Set-A (Entered)
Data Set-B (Entered)
1 3414.693 45.146
2 4257.599 50.643
3 3198.637 43.737
4 4484.848 52.125
5 3762.620 47.415
6 4794.134 54.142
7 4144.741 49.907
8 4641.714 53.148
9 3983.888 48.858
Line
Least squares fit
Equation help
Interpolation of Data 1
Interpolated X values
36°C
Haze
Search
ENG
IN
22:54
14-06-2023

5. Result and Discussion

Objective 1:

To optimize the process parameters critical to the fabrication process. It discusses the selection of the melting temperature and melting time required to generate impermeable hydrophobic barriers.

Properties of paper for microfluidic applications: surface characteristics, surface area, capillary flow rate, pore size, porosity and thickness. The selection of the grade of paper depends on the application of the assay. Formation of paper plates in filter paper prevented spreading of the spot over a large surface. Forming confined spaces also allowed uniformity in both area and tint of the spot, thus enabling the estimation of the concentrations from the intensity of coloration. Paraffin wax was suggested for the formation of water-repellent zones on filter paper due to its general inertness to chemical reagents and its practicality for the formation of diverse patterns.

We used Whatman no. 1 chromatography paper in most of this study because it is hydrophilic, homogeneous, pure, reproducible, biocompatible, and available. It is also relatively inexpensive. It is available in sheets of 460 mm × 570 mm. Filter paper was impregnated with paraffin to demonstrate preferential elution of pigment mixtures.

We used a bronze stamp to print a wax in the filter paper. The stamp dispenses wax from wax coated sheet beneath the filter paper giving imprints of apex on the surface of the paper, where they cool and solidify instantaneously without further spreading. We used a digital hot plate because it provides a flat, uniformly heated surface for heating the paper. Other heat sources, such as ovens or heat guns, can also be used for wax patterning.

5.1 Optimized Time and Temperature:

The area over which the paraffin wax spreads could be controlled by varying the temperature of the printing tool, the pressure, the time of contact and the pore size of the filter paper. It was observed that the matrix in which the analyte is to be detected has not

perfectly formed the hydrophobic barrier at temperature 60°C and 70°C with pressing of stamp time for 30s, 45s and 60s, but at temperature 75°C with pressing of stamp time for 45s completely impermeable hydrophobic barrier with a hydrophilic zone enclosed inside it.

Temperature and time optimization to obtain desired reservoirs:

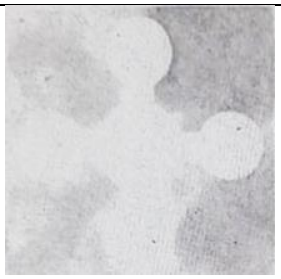
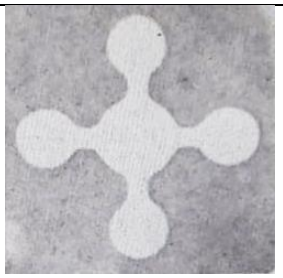
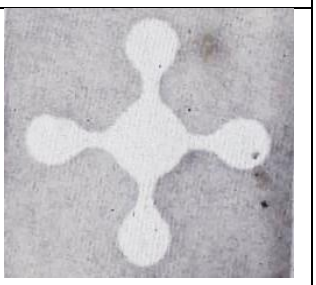
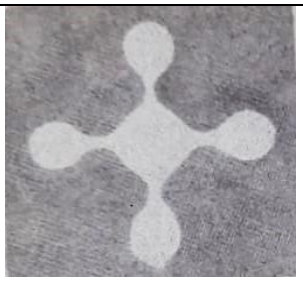
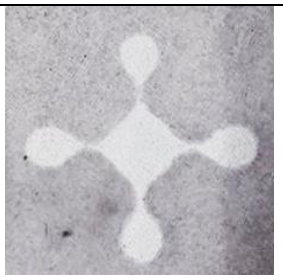
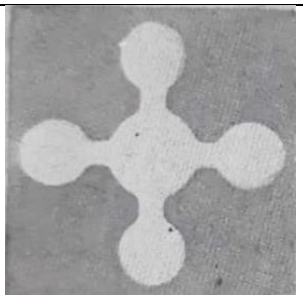
Temp & Time	**30secs**	**45secs**	**60secs**
At 65°			
At 70°			
At 75°			

Figure 13. Stamping was performed at different time and temperature to get the desired reservoirs. It was stamped at 60°C, 70°C and 75°C for 30s, 45s and 60s. The optimised time and temperature for stamping is at 75°C for 45s.

5.2 Device Design:

The paper-based microfluidic device is fabricated with a piece of filter paper, wax and a stamp. The device is consisting of four detection reservoirs and a central sample loading zone connected by distribution channels. All distribution channels are of same length and width and all detection zones have the same diameter to ensure the solution spotted in the circle zone flows equally along all four channels and into the detection zones. Thus the detection reservoirs contain equal amounts of ninhydrin after adding the ninhydrin solution in the device. The three detection reservoirs are spotted with various concentrations of glutamic acid as standard amino acid.

Objective 2:

Determination of the Total Amino Acid Content in blood serum samples using Paper based microfluidics.

The amino acids in the serum sample were determined by the procedure described above. The colorimetric Ninhydrin test was performed and with increasing concentration of amino acids the colour intensity of colorimetric test was increasing as shown in Figure 19. The image of the colorimetric assay for both standard and serum samples were scanned and stored in JPEG format (Figure 20 and 22). The JPEG images were opened with ImageJ software in RGB colour format. The image was then inverted and the mean grey values in detection zones were obtained by subtracting the blank value. Data were imported into GraphPad Prism to obtain a linear correlation (Equation 1) and a standard graph between mean grey intensity and amino concentrations (Figure 21). The mean grey intensity of serum samples was then interpolated in standard graph to obtain the concentration of amino acids (Table 2)

5.3 Colour intensity:

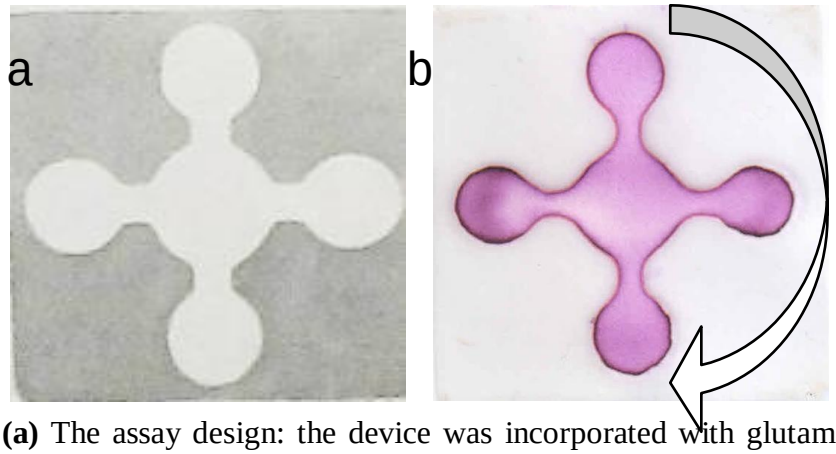


Figure 14. (a) The assay design: the device was incorporated with glutamine (10 µl of concentration 1 mg/ml) at the four reservoirs after being fabricated (grey region represents the hydrophilic area of the device); **(b)**ninhydrin solution was introduced from central inlet zone; it penetrated into each detection zone and triggered different colour changes (violet blue colour). The arrow in figure b shows the increasing amino acid content.

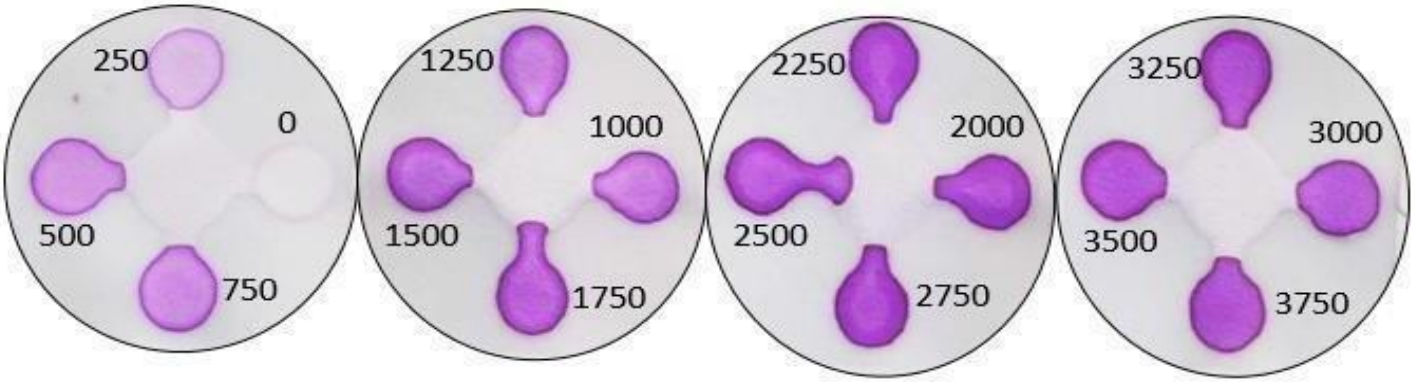


Figure 20. Image showing results of amino acid assay on the paper based device with varied concentrations.

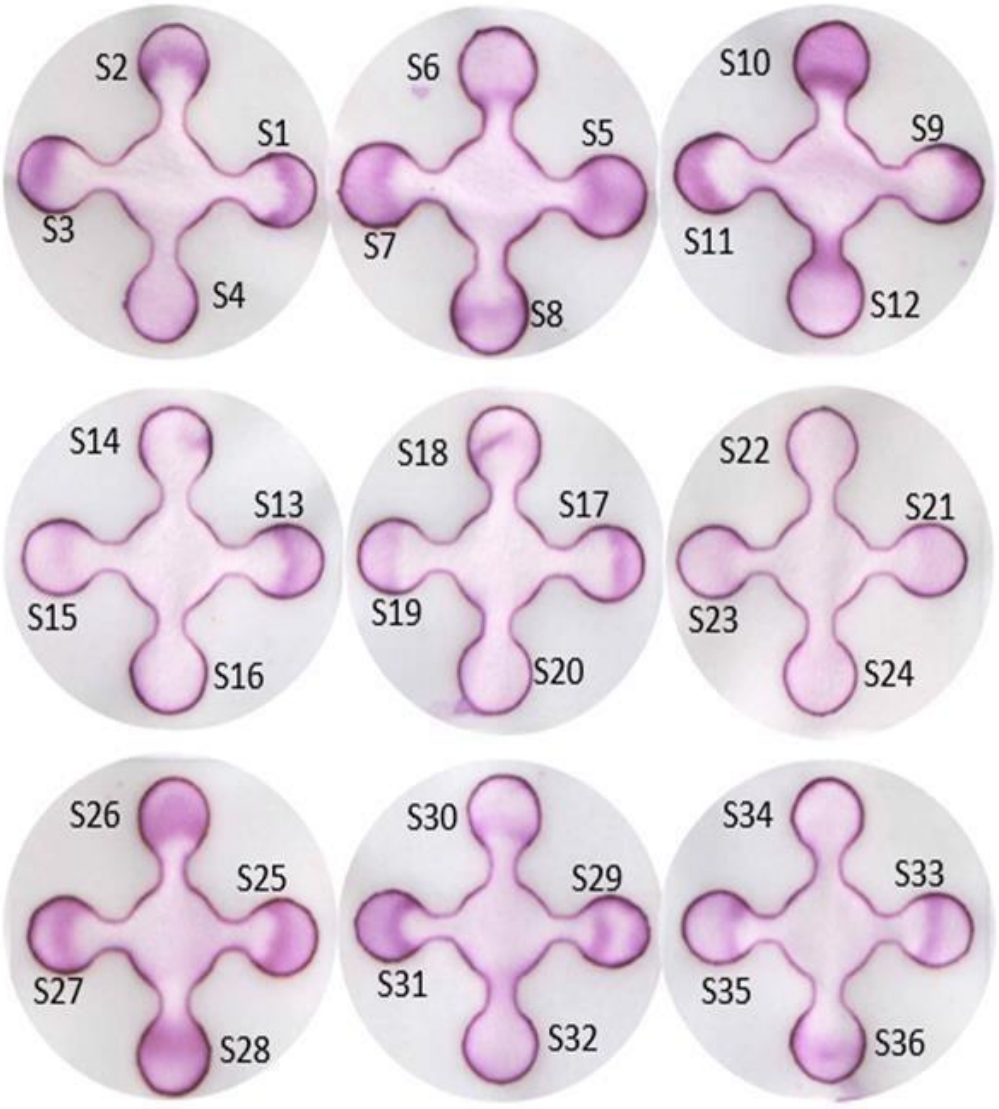


Table 1. Mean grey value varies as a function of glutamic acid concentration obtained from data of figure 20.The mean grey values were obtained by subtracting values of mean provided by the ImageJ software with the blank value.

S.no.	Concentration (µg/ml)	Mean grey value
1.	0	0
2.	250	18.132
3.	500	34.199
4.	750	38.643
5.	1000	51.537
6.	1250	53.788
7.	1500	64.735
8.	1750	67.328
9	2000	80.83
10.	2250	78.387
11.	2500	69.18
12.	2750	80.816
13.	3000	63.414
14.	3250	82.386
15.	3500	68.481
16.	3750	72.75
17.	4000	
18.	5000	49.65
19.	6000	65.87

20.	7000	69.87
21.	8000	72.13
22.	9000	86.71
23.	10000	111.41
24.	11000	116.78
25.	12000	124.76
26.	13000	132.65
27.	14000	140.67
28.	15000	140.76

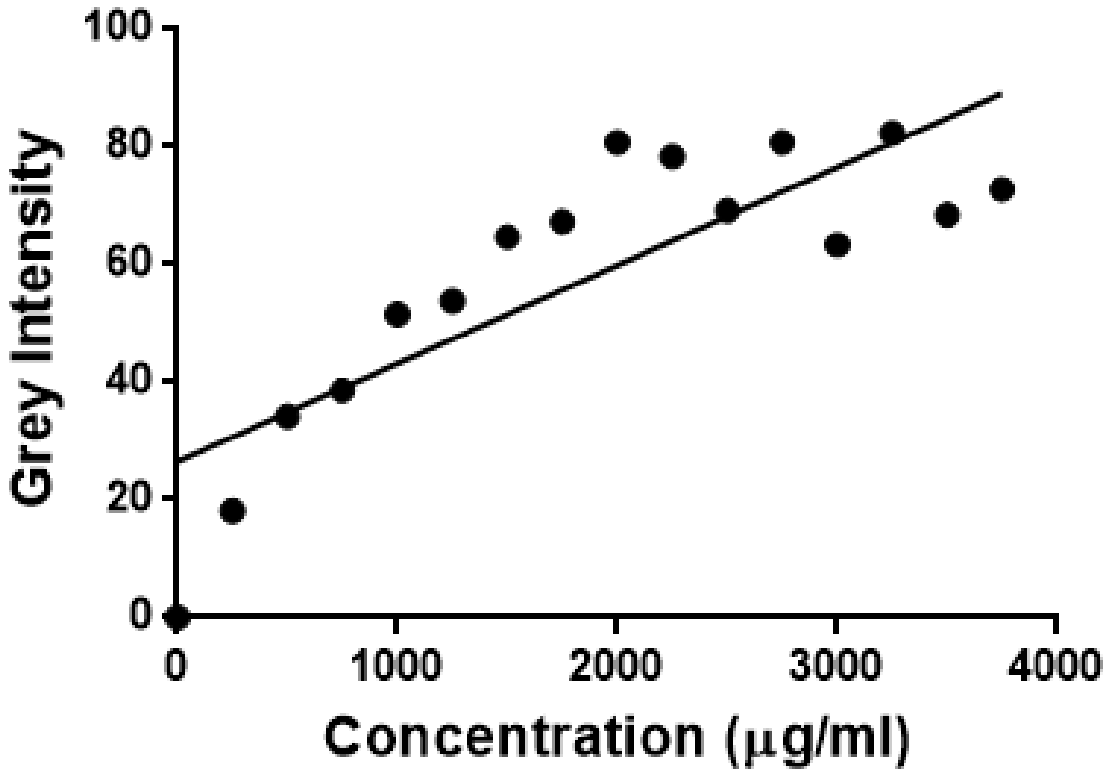
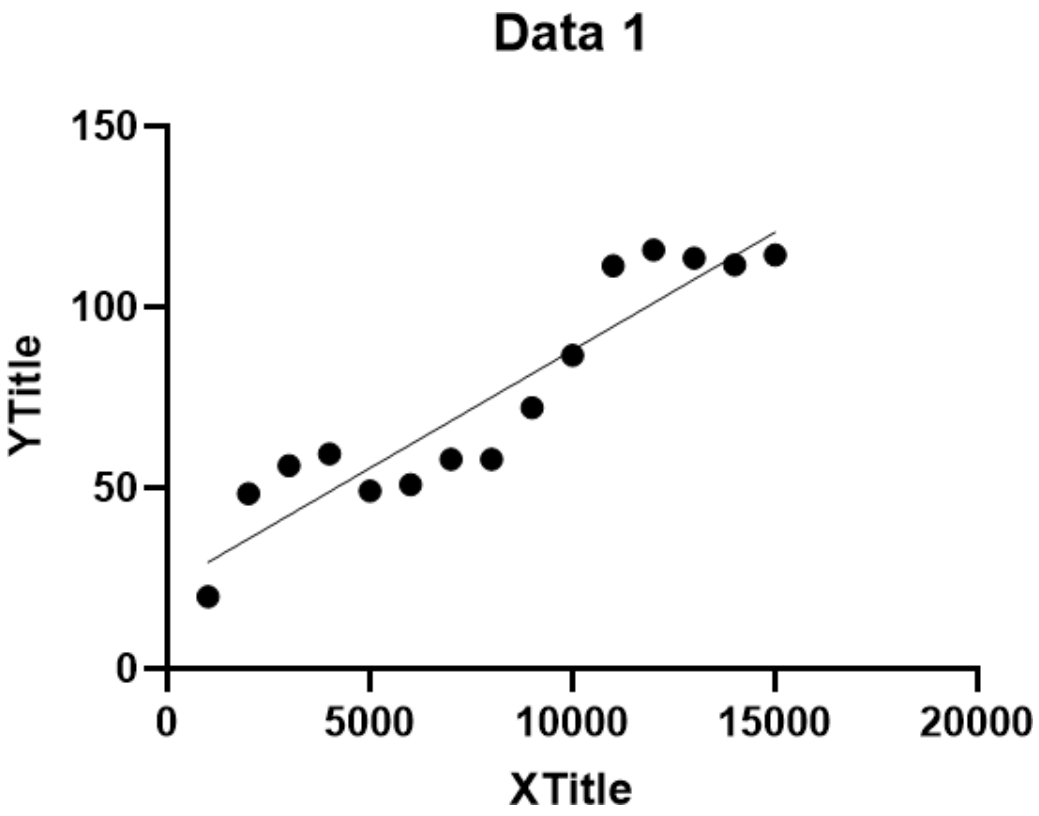


Figure 15. Graph plot of concentration against mean grey value of samples.



Straight line equation:

Equation 1- Y = 0.01668*X + 26.52

The graph was plot against concentration to obtain the straight line of glutamic acid standards. Straight line equation was obtained by GraphPad Prism.

Table 2. As the result was obtained when tested with ninhydrin as shown in (figure 20) these images were then scanned using printer scanner and was uploaded on ImageJ for analyses, obtained the computed values providing the mean values of all the samples and mean grey value was calculated by subtracting the highest mean value among the samples. Now as the equation for straight line was provided by GraphPad Prism (equation 1). We knew the values of Y (mean grey value of samples) and thus could calculate the value of X (concentration of samples).

S.No.	Samples	Concentration (µg/ml)	Mean g value
1	S1	1053.715	44.091
2	S2	787.1865	39.646
3	S3	1227.544	46.99
4	S4	56.67589	27.463
5	S5	717.4514	38.483
6	S6	939.1288	42.18
7	S7	571.8049	36.054
8	S8	182.7749	29.566
9	S9	1222.866	46.912
10	S10	3294.535	81.462
11	S11	1524.413	51.941
12	S12	1580.776	52.881
13	S13	1053.715	44.091
14	S14	787.1865	39.646
15	S15	1545.879	52.299
16	S16	956.098	42.463
17	S17	1126.149	45.299

18	S18	116.0978	28.454
19	S19	337.3556	32.144
20	S20	571.8049	36.054
21	S21	1602.602	53.245
22	S22	1204.398	46.604
23	S23	1802.214	56.574
24	S24	523.0563	35.241
25	S25	1077.88	44.494
26	S26	922.5195	41.903
27	S27	157.4711	29.144
28	S28	115.5581	28.445
29	S29	1135.443	45.454
30	S30	838.3337	40.499
31	S31	722.368	38.565
32	S32	1134.903	45.445
33	S33	615.8165	36.788
34	S34	1085.075	44.614
35	S35	1126.149	45.299
36	S36	1515.239	51.788

S.NO	SERUM SAMPLES	CONCENTRATION(µg/ml)	MEAN GREY VALUE
1	**S1**	**3414.693**	**43.737**
2	**S2**	**4257.599**	**50.643**
3	**S3**	**3198.637**	**45.146**
4	**S4**	**4484.848**	**52.125**
5	**S5**	**3762.620**	**47.415**
6	**S6**	**4794.134**	**54.142**
7	**S7**	**4144.741**	**49.907**
8	**S8**	**4641.714**	**53.148**
9	**S9**	**3983.888**	**48.858**
10	**S10**	**4567.876**	**50.654**

6. Conclusion

We described a method for the determination of total amino acids in blood samples by using a simple paper-based microfluidic device. This device is easy to fabricate and could be applied to microfluidic teaching especially in those less industrialized regions.

A fast (10 s), cheap and simple method to produce paper-based microfluidic devices by contact stamping using a bronze stamp has been developed. Paraffin wax ink can form effective hydrophobic barriers that constrain the diffusion of water within the boundaries of the wax pattern. Capillary flow rate is an important parameter for paper-based microfluidics. It is defined as the migration speed of a sample front moving along the length of the membrane strip. The capillary flow rate is critical in achieving consistent sensitivity depending on the location of the detection line/ zone. Whatman filter paper grade 1 has been used to produce the microfluidic device because its porous, high flow rate and capillary action.

Compared to other reported paper-based microfluidic device fabrication technologies, contact stamping is capable of providing an easy way to produce paper-based microfluidic structures without sophisticated infrastructural requirements. For instance, in developing countries replicas of paper-based microfluidics for diagnostic purpose could be produced by non-especially trained staff employing the contact stamping technique, bearing in mind that only a hot plate, paraffin wax, filter paper and a bronze stamp with the designed features need to be provided.

Research on paper-based microfluidic devices is still at an early stage; significant research efforts will be needed in this field to nurture it into a more matured platform technology in diagnostic, point-of-care (POC), and environmental monitoring applications. Approaches towards instrument free detection of amplicons along with the development of PCR-on-paper or nitrocellulose, whole cell detection on paper/ nitrocellulose, tissue-on-paper and all-in-one assays will be important contributions to the field. Furthermore, the quality of paper after long-term storage, and time-consuming sensors (e.g. full colour development) require further improvements. Having performance data does not always yield efficacy after deployment. Possible trials must establish the feasibility and cost-effectiveness after scaling up. The ultimate test in the realisation of

paper-based microfluidics depends on experts in the commercial diagnostics industry and this may require evidence for acceptance. One challenge for the academic community is to justify the feasibility of the proposed technologies. The lateral-flow format is the only ubiquitous, universally applicable platform that can be utilised for simple, qualitative, low cost point-of-care applications, while also having enough capability to be utilised in highly sensitive, fully quantified, multiplexed assays. In developing world markets, some applications may be found, but even there purchasing decisions tend to go through the tender process from organizations like the WHO or other large foundations and governmental agencies, who look for performance as well as price. It may be expected that low cost on its own will not be enough to achieve market penetration. The value of diagnostics can only be realised if they meet the performance requirements, manufactured at high-volume and actions can be taken towards treatment.

7. REFERENCE

1. Ali Kemal Yetisen, Muhammad SafwanAkram and Christopher R. Lowe,"Paper-based microfluidic point-of-care diagnostic devices" RSC Publishing. DOI: 10.1039/c3lc50169h
2. W. Martinez, S. T. Phillips, M. J. Butte, and G. M. Whitesides (2007) "Angew. Chem., Int. Ed." 46(8), 1318.
3. W. Martinez, S. T. Phillips, and G. M. Whitesides (2008) "Proc. Natl. Acad. Sci. U.S.A." 105(50), 19606.
4. Abrams, H.H. Bau (2011) "Point-of-care oral-based diagnostics", Oral Dis. 17.
5. AkyaziTugce, Basabe-Desmonts Lourdes, Benito-Lopez Fernando "microfluidic paper-based analytical devices towards Commercialisation" doi/10.1016/j.aca.2017.11.010.
6. Rozand (2014) "Paper-based analytical devices for point-of-care infectious disease testing", Eur. J. Clin. Microb. Infect. Dis. 33.
7. Choi Young-Soo, Im Min Kyu, Lee MiRha, Kim Cheol Soo, Lee Kyeong-Hwan "Highly sensitive enclosed multilayer paper-based microfluidic sensor for quantifying proline in plants" doi/10.1016/j.aca.2020.01.038.
8. Dungchai et al. "A low-cost, simple, and rapid fabrication method for paper-based microfluidics using wax screen-printing"
9. Delaney, J. L., C. F. Hogan, J. Tian, and W. Shen (2011) "Anal. Chem." 83(4), 1300.
10. A. Bruzewicz, M. Reches, and G. M. Whitesides (2008) "Anal. Chem." 80(9), 3387.
11. M. Fenton, M. R. Mascarenas, G. P. Lo´pez, and S. S. Sibbett, (2008) "ACS Appl. Mater. Interfaces" 1(1), 124.
12. Carrilho, A. W. Martinez, and G. M. Whitesides (2009) "Anal. Chem." 81(16), 7091.
13. Emanuel Carrilho et al. "Understanding Wax Printing: A Simple Micropatterning Process for Paper-Based Microfluidics"
14. Chitnis, Z. Ding, C.-L. Chang, C. A. Savran, and B. Ziaie (2011)," Lab Chip" 11(6), 1161.
15. JuusoOlkkonen et al. "Flexographically Printed Fluidic Structures in Paper".
16. J. Hu, S. Wang, L. Wang, F. Li, B. Pingguan-Murphy, T.J. Lu, F. Xu (2014) "Advances in paper-based point-of-care diagnostics", Biosens. Bioelectron. 54.
17. K. Abe, K. Suzuki, and D. Citterio (2008) "Anal. Chem." 80(18), 6928.
18. K. Abe, K. Kotera, K. Suzuki, and D. Citterio (2010) "Anal. Bioanal. Chem." 398(2), 885.
19. Kentaro Yamada et al. "Paper-Based Inkjet-Printed Microfluidic Analytical Devices"
20. Li Xu, Ballerini David R. and Shen Wei "A perspective on paper-basedmicrofluidics:
21. Current status and future trends" doi.org/10.1063/1.3687398.

22. Martinez, A. W., S. T. Phillips, E. Carrilho, S. W. Thomas, H. Sindi, and G. M. Whitesides, (2008) "Anal. Chem." 80(10), 3699.
23. Martinez, A.W., S.T. Philips, G.M. Whitesides, E. Carrilho (2010) "Diagnostics for the developing world: microfluidic paper-based analytical devices", Anal. Chem. 82.
24. Martinez Andres W "Microfluidic paper-based analytical devices: from POCKET to paper-based ELISA" doi.org/10.4155/bio.11.258.
25. NishatSumaira, TurabJafry Ali , W.Martinez Andres, Awan Rabbi Fazli "Paper-based microfluidics: Simplified fabrication and assay methods" doi.org/10.1016/j.snb.2021.129681
26. Petti, C.A., C.R. Polaga, T.C. Quinn, A.R. Ronald, M.A. Sande (2006) "Laboratory medicine in Africa: a barrier to effective health care", Clin. Infect. Dis. 42.
27. Paper-based microfluidics: Simplified fabrication and assay methods – ScienceDirect.
28. Quantitative Determination of Total Amino Acids in Tea Using Paper Microfluidics and a Smartphone Laboratory Methods in Microfluidicsdoi/10.1016/B978-0-12-813235-7.00012-X.
29. R.W. Hart, M.G. Mauk, C. Liu, X. Qiu, J.A. Thompson, D. Chen, D.D. Malamud, W.R.
30. S. Klasner, A. Price, K. Hoeman, R. Wilson, K. Bell, and C. Culbertson (2010) "Anal. Bioanal. Chem." 397(5), 1821.
31. Strong, E.B., Schultz, S.A., Martinez, A.W. et al. Fabrication of Miniaturized Paper-Based Microfluidic Devices (MicroPADs). Sci Rep 9, 7 (2019). doi/10.1038/s41598-018-37029-0.
32. V. Leung, A.-A. M. Shehata, C. D. M. Filipe, and R. Pelton (2010) "Colloids Surf.", A 364(1–3), 16.
33. W. Dungchai, O. Chailapakul, and C. S. Henry (2011), "Analyst (Amsterdam)" 136(1), 77.
34. X. Li, J. Tian, G. Garnier, and W. Shen (2010) "Colloids Surf.", B 76(2), 564.
35. X. Li, J. Tian, T. Nguyen, and W. Shen (2008) "Anal. Chem." 80(23), 9131.
36. X. Li, J. Tian, and W. Shen (2010) "Cellulose" 17(3), 649.
37. Yanyan Xia et al. "Fabrication techniques for microfluidic paper-based analytical devices and their applications for biological testing: A review".
38. Y. Lu, W. Shi, L. Jiang, J. Qin, and B. Lin (2009) "Electrophoresis" 30(9), 1497.
39. Yager, P., T. Edwards, E. Fu, K. Helton, K. Nelson, M.R. Tam, B.H. Weigl (2006) "Microfluidic diagnostic technologies for global public health", Nature 442.
40. Yager, P., G.J. Domingo, J. Gerdes, (2008) "Point-of-care diagnostics for global health", Annu. Rev. Biomed. Eng. 10.
41. Zheng, W., Wang, K., Xu, H. et al. Strategies for the detection of target analytes using microfluidic paper-based analytical devices. Anal BioanalChem 413, 2429–2445 (2021). https://doi.org/10.1007/s00216-021-03213-x.
42. https://www.elveflow.com/microfluidic-reviews/general-microfluidics/paper-microfluidic-devices-a-review-2017/#_ftn1.