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Διπλωματική Εργασία

Σχεδιασμός Συσκευής Για την Συλλογή Πρωτεΐνης από Εκπνεόμενο Αέρα

Thesis: Design of a Protein Collecting Device from Exhaled Air

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Καθηγητής ΕΜΠ

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Παναγιώτης Ιορδανίδης

Abstract

The technological developments and the COVID-19 pandemic have increased the demand for better, less invasive diagnostic tools and prompted the public to be more interested in monitoring its health. The answer to those needs can be breath analysis, which is a non-invasive diagnostic method that can be used by people from almost all age groups. There are many commercial breath analyzers that detect different substances, but none of them detects protein. At the same time, the field of proteomics can play a crucial role in diagnosing and tracking the health state of a person. The aim of this project was to create a device that can be used as a test to collect protein from exhaled air while being as user-friendly and simple as possible.

This study started with the conceptual design of the product, which was based on the previous specifications. Afterwards, the device was 3D-printed in order to check the quality of the parts of the device and to determine the most suitable design. The final design was based mostly on the user experience while taking into account many other factors, such as the limitations of the 3D printer and the repetitiveness of quality. Next, a part of the device was 3D printed in medical resin type I and the other parts were printed in ABS like resin, and they got sterilized. Then, the product was used in order to perform our experiments. In the first experiments, the goal was to detect protein in the samples by observing color change and determining the external factors that could impact the results of the experiments. The next experiments had BCA analysis and the aim was to detect protein in the samples for different numbers of exhaled breaths, to try to quantify the concentration of protein in the samples and to determine what number of breaths is suitable and should be set in order to have feasibility. After a standard number of exhaled breaths was set, the BCA analysis was performed numerous times and it was evident that there was protein in the exhaled air, and its concentration was approximately measured, proving the feasibility of the test.

Περίληψη

Οι τεχνολογικές εξελίξει και η πανδημία του κορονοϊού έχουν αυξήσει την απαίτηση για καλύτερα, λιγότερο επεμβατικά διαγνωστικά εργαλεία και ώθησαν το κοινό να ενδιαφέρεται παραπάνω για την παρακολούθηση της υγείας του. Η απάντηση σε αυτές τις ανάγκες μπορεί να είναι η ανάλυση αναπνοής, η οποία είναι μία μη επεμβατική διαγνωστική μέθοδος που μπορεί να χρησιμοποιηθεί από ανθρώπους σχεδόν σε όλων των ηλικιακών ομάδων. Υπάρχουν πολλοί εμπορικοί αναλυτές αναπνοής που ανιχνεύουν διαφορετικές ουσίες, αλλά κανένας από αυτούς δεν ανιχνεύει πρωτεΐνη. Ταυτόχρονα, ο τομέας της πρωτεομικής μπορεί να παίξει ένα κρίσιμο ρόλο για την διάγνωση και την παρακολούθηση της κατάστασης της υγείας ενός ατόμου. Στόχος αυτής της εργασίας ήταν να δημιουργήσει μια συσκευή που μπορεί να χρησιμοποιηθεί ως τεστ για την συλλογή πρωτεΐνης από τον εκπνεόμενο αέρα αλλά και να είναι όσον τον δυνατόν πιο φιλική προς το χρήστη και απλή γίνεται.

Αυτή η εργασία ξεκίνησε με τον αρχικό σχεδιασμό τους προϊόντος ο οποίος βασίστηκε στις προηγούμενες προδιαγραφές. Μετέπειτα, η συσκευή εκτυπώθηκε σε 3Δ-εκτυπωτή προκειμένου να ελεγχθεί η ποιότητα των τεμαχίων της συσκευής και να καθοριστεί ο καταλληλότερος σχεδιασμός. Το τελικό σχέδιο βασίστηκε κυρίως στην εμπειρία του χρήστη, λαμβάνοντας παράλληλα υπόψιν πολλούς άλλους παράγοντες όπως οι περιορισμοί του 3Δ-εκτυπωτή και η επαναληψιμότητα της ποιότητας. Στην συνέχεια, ένα κομμάτι της συσκευής εκτυπώθηκε με μια βιοσυμβατή ρητίνη τύπου I και τα άλλα μέρη εκτυπώθηκαν με μια ρητίνη φωτοπολυμερούς τύπου ABS και αποστειρώθηκαν. Κατόπιν, το προϊόν χρησιμοποιήθηκε για να πραγματοποιηθούν τα πειράματα. Στα πρώτα πειράματα, ο στόχος ήταν η ανίχνευση πρωτεΐνης στα δείγματα παρατηρώντας αλλαγή του χρώματος και ο καθορισμός των εξωτερικών παραγόντων που μπορούν να επηρεάσουν τα αποτελέσματα των πειραμάτων. Τα επόμενα πειράματα είχαν την μέθοδο BCA (βικινχρωμικό οξύ) και σκοπός ήταν να ανιχνευθεί πρωτεΐνη στα δείγματα για διαφορετικούς αριθμούς εκπνοών, να γίνει μια προσπάθεια ποσοτικοποίησης της συγκέντρωσης πρωτεΐνης που διαθέτουν τα δείγματα, και να καθοριστεί ο αριθμός εκπνοών που είναι κατάλληλος και πρέπει να τεθεί ως στάνταρ για να λειτουργήσουν τα πειράματα. Αφού ένας στάνταρ αριθμός εκπνοών είχε τεθεί, η μέθοδος BCA χρησιμοποιήθηκε πάλι πολλαπλές φορές και έγινε φανερό ότι υπήρχε πρωτεΐνη στον εκπνεόμενο αέρα ενώ η συγκέντρωση της υπολογίστηκε κατά προσέγγιση, αποδεικνύοντας ότι το τεστ λειτουργεί.

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1. Introduction

1.1 Reasons why breath sampling is important

Breath sampling has better attributes than current sampling methods used for biomarkers for various reasons. First of all, more than 3000 volatile organic compounds (VOCs) and respiratory droplets from the lungs as well as the airways can be found in exhaled breath [1]. Both VOCs and respiratory droplets can potentially be breath biomarkers, hence contain information about the health of a person. Breath sampling is also non-invasive. It doesn't cause any pain or contains any risk for the user, in comparison to other methods such as blood collection and urine collection which although well-established are often uncomfortable for the user. Another unique feature of breath collection lies in its nature. The human body produces exhaled breaths as a waste product in a constant rate and in large quantities. That leads to having no limits in collection regarding sampling size and frequency. Other traditional methods like blood, urine or sweat collection have limited sample size and frequency because of the nature of their samples. Additionally, the breath sample can contain important biological information because it reflects metabolic activity. The first stages of a disease are often displayed in the metabolism and many breath biomarkers are metabolic products, hence making them highly sensitive because a change in a gene can be amplified in metabolism. Some diseases can have metabolic impact without necessarily affecting the genetic complement of a cell. As a result, a breathing sample can be a representative sample for diseases. In addition, mostly genetic alterations that are usually expressed on later stages of a disease are being detected by current biopsy techniques. That makes breath sampling suitable for early detection, diagnosis, prognosis and precision medicine. Furthermore, the health state of the user can be depicted through breath collection as it allows full body monitoring. The blood circulates the whole body approximately every minute while carrying VOCs produced from various sources throughout the whole body. Therefore, it's possible to collect biomarkers from various diseases and potentially collect more than just one biomarker. Finally, one factor that should not be underestimated is the accessibility of breath sampling. It can be used without having any medical knowledge or requiring the presence of trained personnel as opposed to other sampling methods.

1.2 Breath collection methods

Regarding the methods for breath collection, it is important to note that although there are several methods for collecting and analyzing breath, as of now there is not a standardized exhaled breath collection method. As a result, there is difficulty in comparing results between studies. Nonetheless, it is crucial to have standardized a breath sampling procedure during experiments in order for the results to be repeatable and due to the fact that the procedure itself has a significant impact on the results.

Many studies and research centers are using late respiratory sampling, also known as the “drinking straw” method [2]. The sample is collected in a tube and the user is asked to exhale partially and then blow the rest of the breath through the drinking straw inside the tube in a single motion. The advantage of this method is considered to be the fact that by discarding an initial proportion of the exhaled air, the levels of exogenous VOCs are minimized and the more endogenous VOCs are captured, since the air at the end of the breath cycle is captured. Another popular method is the “flute” method [2]. In that method, the user is asked to take a deep breath and blow in a single continuous exhalation into the tube. There are concerns regarding the ability of the user to actually cooperate with the first method, especially for people from younger age groups. Pressure sensors could be equipped for this predefined stage of exhalation [3|4] but that can increase both costs and complexity. For these reasons, the second method can be preferred and be more suitable in certain cases. An example of a tube-based breath sampler is depicted in the following figure 1.1

Figure 1.1: A tube-based breath sampler [5]

Apart from tubes, polymer bags are also often used in breath sampling due to their durability and low costs. Those bags can also be reused provided that they are suitably cleaned. The next figure 1.2 is an example of that type of bag.

Figure 1.2: A breath collection bag [6]

1.3 Breath analyzers

Despite all the advantages that breath analysis has, except for breath analyzers that detect alcohol levels, few analyzers have actually attained a high level in terms of technological readiness [7]. In fact, diagnostic applications are sometimes restricted in the form of chromatography tests and often require expensive equipment for analysis.

However, there are various devices that are being developed by companies and institutions around the world. Some have even been patented but some of them are intended to be used for research only.

An example can be the patent number:11298046 which is a device for breath sampling in the form of a mask that is placed over the user's mouth and nostrils to collect breath exhaled. It can detect movement of the mask portion and signal if its misplaced. It is important to mention that a number of patents have been assigned to the same company that holds that patent related to that device, systems and the methods surrounding the sampling procedure, like the patent number: 11617521, which is a method for collecting different breath samples or even fractions of them on a single device in which the first and second sample are captured in a spatially separated manner. It's important to mention that these patents concern a product that is for research only and it is not being used in established diagnostic procedures.

Another example is the patent number: 9011348. This patent is a sampling device with a closed loop system in order for the extracted air to be sampled into an analysis device. The closed loop method reduces the possible contaminants in that sample.

Moreover, there is a device and a method for rapidly and accurately identifying and quantifying analytes in mixture that has the patent number: 8288727. The product consists of an ultra-sensitive cavity enhanced spectrometer as well as devices for data collection and analysis. On the other hand, the method employs a database that contains the cross section of numerous analytes in order to determine the composition of a sample in a numerical way.

Apart from the devices that are patented. There are some commercial breath analyzers used for specific cases such as smoking cessation, CO poisoning, asthma management, gastrointestinal disorders and food intolerance [8].

At the same time, in some institutions, an effort is made in order to develop cheap and portable breath analyzers with some of them being in the early stages of being a product.

It is essential to also mention that the US FDA or EU EMA have given medical or regulatory approval to a number of breath-based tests and there are some breath-based tests that are recommended by professionals and thus having routine use [9]. Those tests are either focused on exogenous or endogenous compounds. Examples of this kind of tests for endogenous compounds are the Capnography test, which is used for breathing and targets carbon dioxide (CO_2) and the F_ENO test for asthma which targets the Nitrogen Oxide in a fraction of an exhaled breath. Examples of the breath-based tests for exogenous compounds are the Hydrogen breath test for lactase deficiency with Hydrogen as the target and the very well-known breath alcohol test for alcohol intake that targets Ethanol. An example of equipment used for breath-based tests is shown in figure 1.3.

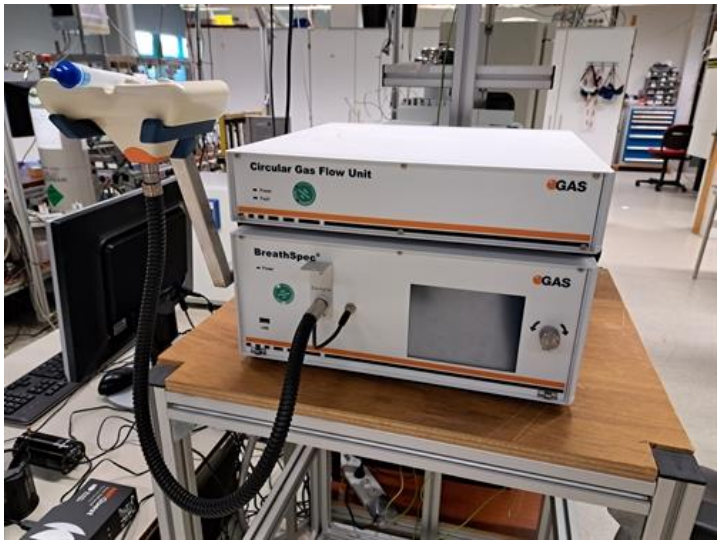

Figure 1.3: GC-IMS system [10]

1.4 What do these solutions lack

Although some of these solutions are brilliant and advanced, they have some disadvantages that cannot be ignored. First of all, although the previous breath-based tests mentioned are well established or approved, they require expensive, advanced or even bulky equipment to be performed and analyzed, with the exception of the alcohol test. They also require trained personnel to supervise the procedure. Moreover, when it comes to the patents, the device for quantifying and identifying analytes can be considered bulky, while trained personnel is essential. The patent with the closed loop system still needs to be coupled with an analysis device, which means that laboratory equipment is necessary. In the first two patents, even though the device is portable and user-friendly, it still requires analysis from a laboratory, meaning more costs and it is used for research only. Another important factor is that most solutions are only analyzing the VOCs in the exhaled breath and not the proteins that can be present. The reason why proteins are so important will be explained further in the next section.

1.5 Reasons why proteomics is important

The methodical and extensive study of proteins, their structure and physiological role or functions is known as proteomics [11|12]. The proteome is a set of proteins produced by a cell, tissue or organism under defined conditions. Proteins are involved in almost every biological process, so a thorough examination of them can shed light on the way these molecules interact, create and sustain a biological system. Proteomics can answer questions such as when and where proteins are expressed, how proteins are modified like post-translational modifications (PTMs) or their movement in different compartments inside the cell [13]. The big disadvantage of genomics is that it cannot give direct measurements of a cellular state or reveal the changes in proteins related to that change. Those changes can give an image of the cell's regulatory network because the cells respond to external changes by regulating their proteins. Proteomics can also give information on the rate that proteins are being produced, degraded or in a steady state, as well as the role of proteins in the metabolic pathways. Before a disease is obvious in the body of a patient, it can be expected that there will be changes in the proteome of affected tissues. Those variations can be analyzed and work as biomarkers to forecast changes in a patient's health or their response to certain medications. That being so, an application can be doctors to check the proteome of a patient's sample in order to see if something wrong is happening and stop health issues from developing or lower the severity of symptoms by prescribing preventative treatment. As a result, disease related mechanisms can be revealed and both prognostic and diagnostic biomarkers can be identified [14]. Consequently, proteomics can help in early diagnosis, prognosis, monitoring disease development and drug development in the form of target molecules.

1.6 The proposal

Considering all this previous information, it becomes evident that there is a need in the market for a user-friendly, simple breath-based test that is based on protein from the exhaled air. For that reason, a device was created which is completely non-invasive and doesn't create any unpleasant feelings to the user. Additionally, it is portable and simple to use by the average user from different age groups. It is also important for the manufacturing costs to be low. Finally, when it will be developed into an end product, it will not require any analysis from specialized laboratories, which means lower costs and faster results. This product will combine the advantages of both breath sampling and proteomics.

Considering the need not to cause any unpleasant feeling while being non-invasive and simple, the device was given the shape of a color crayon. It also partially resembles the appearance of some tobacco products. The first shape is something that the users are familiar with from their childhood and that makes it appealing to both younger and older persons. The device is shown in the figures 1.4 and 1.5

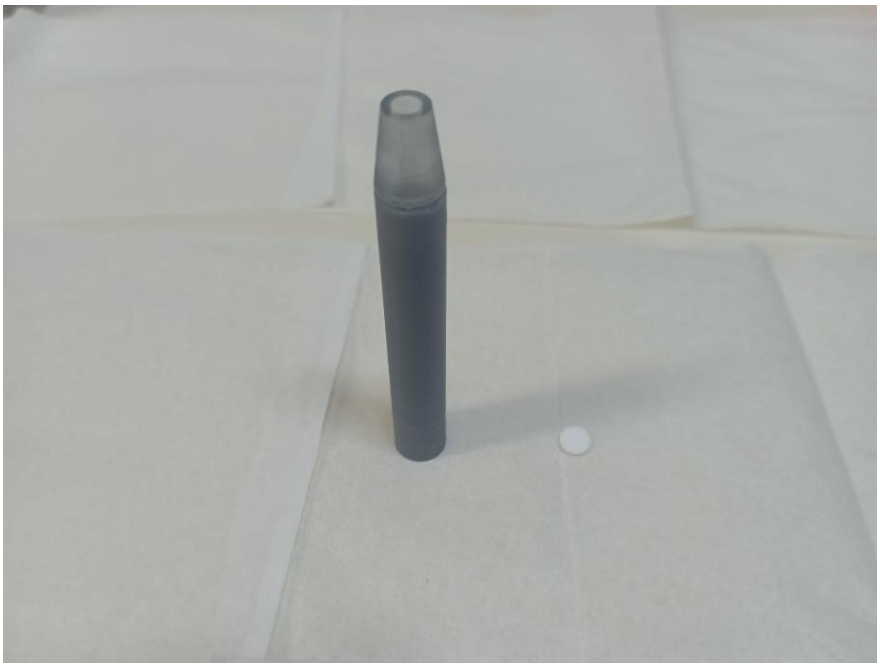

Figure 1.4: The device

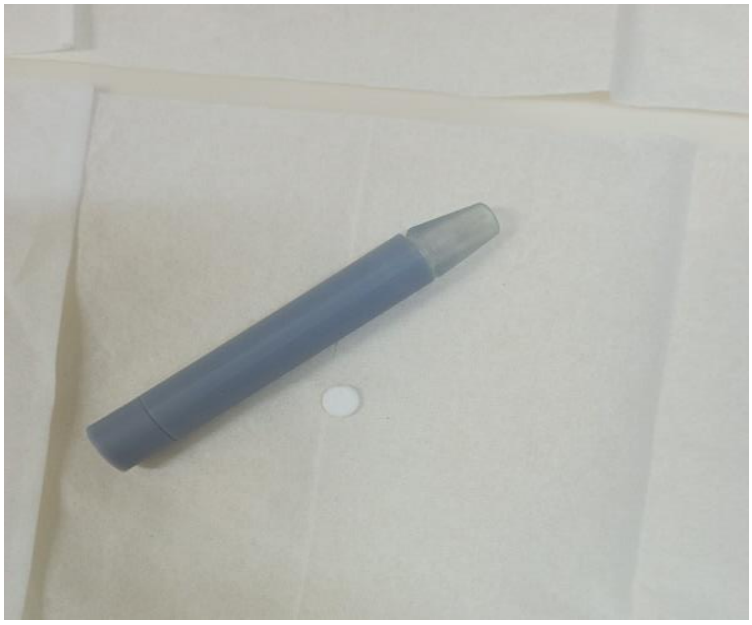

Figure 1.5: The device with the paper filter

The device consists of three parts as depicted in figure 1.6.

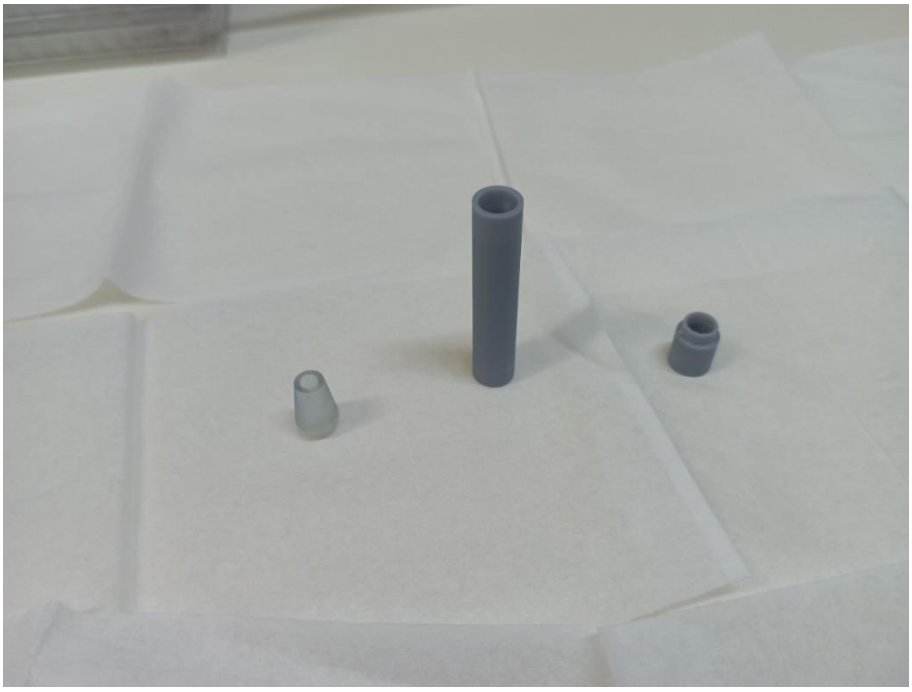

Figure 1.6: The three parts of the device

The paper pad that is shown in figure 1.5 is placed at the bottom of the device. Afterwards, the subject is instructed to blow through the device for a specific number of times. The air passes through the paper pad which serves as a filter. Then, the paper filter is taken from the bottom part and gets analyzed.

2. Design of the device

2.1 Specifications of the device

Before the conceptual design started, it was necessary to state the specifications of the device. The specifications are shown below:

Main design parameter	The device should look like a color crayon in order for the user to find it familiar and feel comfortable using it.
Extra safety measure	The device needs to have an extra safety measure so that there is no risk of the user swallowing the paper filter.
Simplicity	The device needs to be very simple so that it can be used by people from different age groups without difficulty.
Assembling / Disassembling	The device needs to be assembled and disassembled easily. The reason behind this is that the bottom part will need to be taken out easily in order to take the paper filter off and analyze it. Nevertheless, it can be expected that the device will be ready to use when taken out of the package, meaning that it will be already assembled with the paper filter already put in.
Transportation	The device needs to be portable. Furthermore, it should be transportable in a small packing for reduced transportation costs.
Manufacturing	It is crucial for the manufacturing costs to be low. Due to expected high demand, injection molding seems to be the most effective solution time-wise and cost-wise.

A typical color crayon is presented in the figure 2.1.

Figure 2.1: A typical color crayon [15]

A comparison between the device and the color crayon is depicted in the figure 2.2

Figure 2.2: Comparison between the prototype and a color crayon

2.2 Stages of the design

Since the device's primary specifications were user-friendliness and simplicity, it is clear that reaching the final design was not so simple. Much brainstorming, prototyping and testing were performed, each with its respective time.

2.2.1 Conceptual design - Brainstorming

At the start of the design, there were many ideas. The idea of a color crayon-like shape matched the user-friendliness specification and at the same time it was simple and had the advantage that, because of its shape, the top part would obviously be the tip, hence making it obvious for the user how to use it. After the main design was determined, there were other parameters to be decided. The 3D modelling was performed in SolidWorks and at first the idea was for the device to consist of two parts as depicted in the figure 2.3.

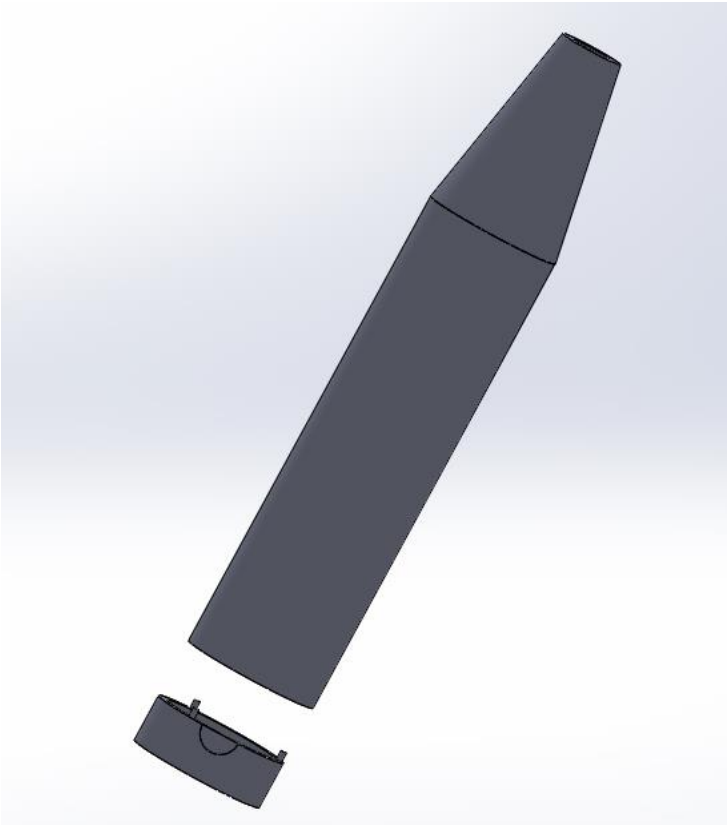

Figure 2.3: The two separate parts of the first concept of the device

This design was quickly discarded though, because the top part of the design needed to be 3D-printed with biocompatible medical resin type I and the other with ABS like resin. The medical resin has a high cost though, so 3D-printing the top part with this type of resin would be very costly and also unnecessary since the user's mouth only touches the top part-tip of the device which was depicted in the figure 1.6. It is also important to mention that because of the different properties of resins, testing and prototyping various versions until finding the final design would be very costly as a consequence of unnecessary use of the expensive resin.

Therefore, it was decided that the device should consist of three parts as pictured in the figure 2.4.

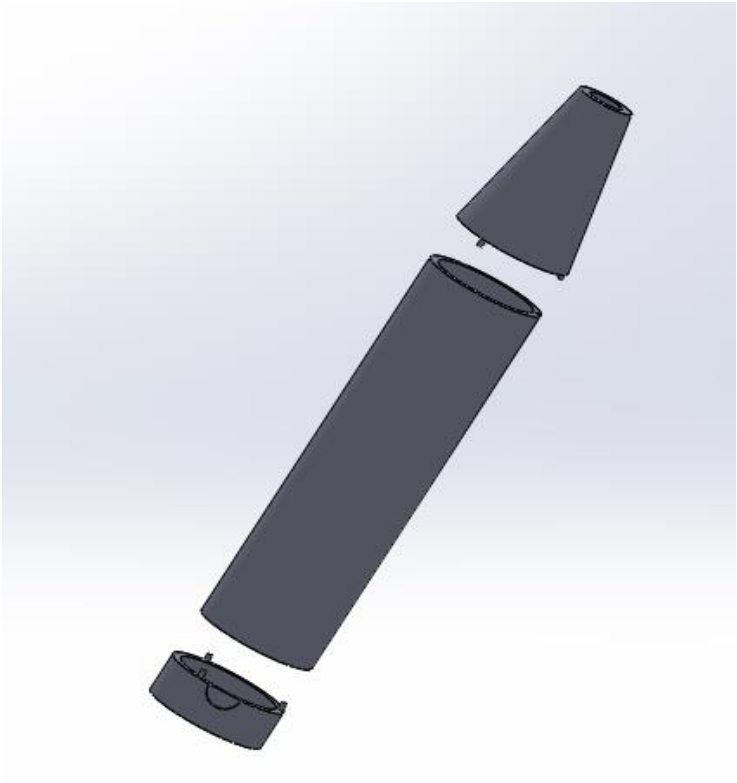

Figure 2.4: The three parts of the first concept of the device

The top part-tip would be 3D-printed with the medical resin and the other two would be 3D-printed with ABS-like resin. That combination is both cost effective and time-effective.

2.2.2 Locking mechanism between the three parts

After it was decided that the device would consist of three parts, the next parameter was which locking mechanism should be used to connect the three parts while taking simplicity and ease of use into account. The first solution was the snap fit mechanism. This solution was depicted already in figure 2.4. A close look of the snap fit mechanism is depicted in the figure 2.5.

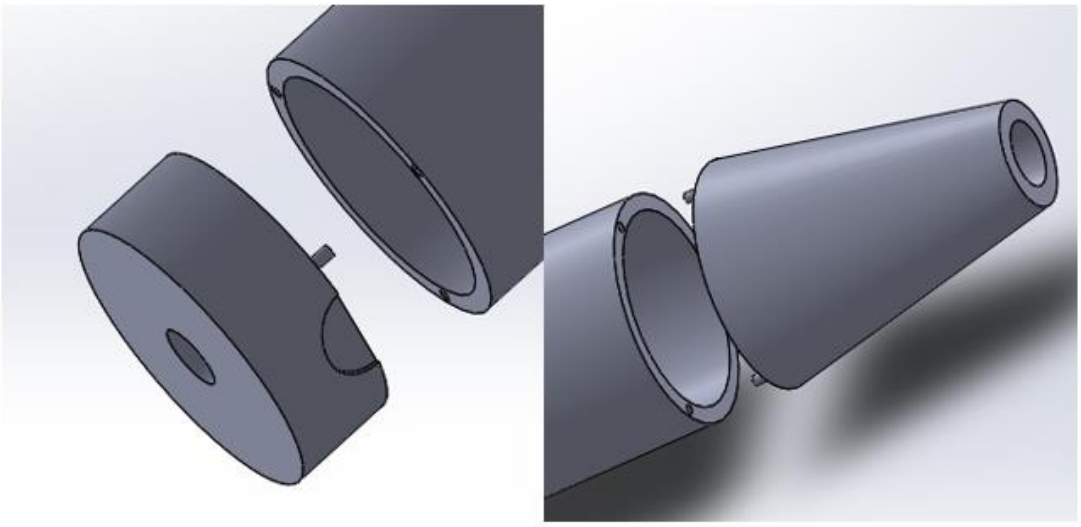

Figure 2.5: The first solution with the snap fit for the device

The bottom part also had a small cavity in order for the user to be able to take it off with ease and analyze the paper filter. This is presented in the figure 2.6.

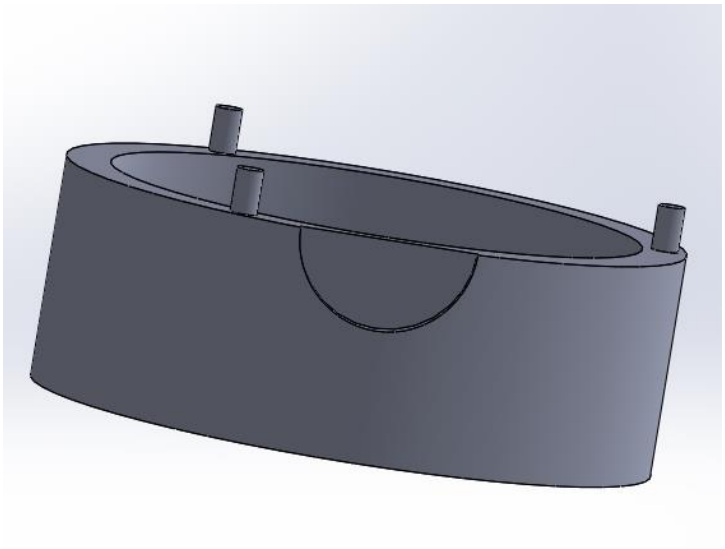

Figure 2.6: The cavity in the bottom part

This solution was rejected because even though the mechanism could potentially work between the two parts made of the same resin (ABS like), it would be difficult for it to work between the part with the medical one and the one with the ABS like. Those resins have different properties, so the snap fit would probably break at the bending moment, making the device not suitable for use.

The next solution was using threads for the parts to connect. This is depicted in the figures 2.7 and 2.8.

Figure 2.7: The second solution with the thread connection of the parts of the device

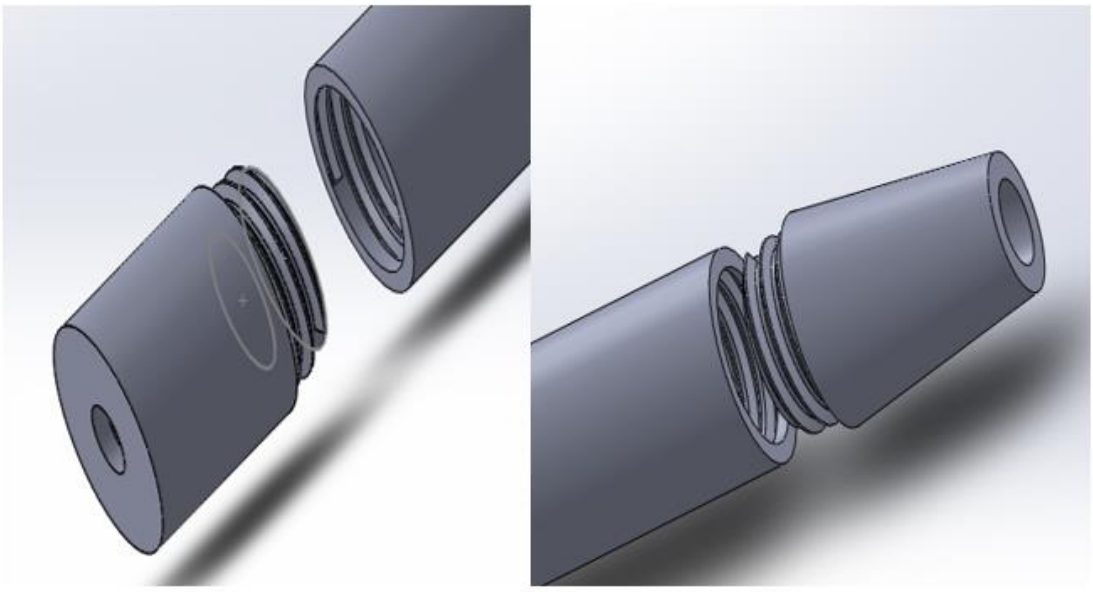

Figure 2.8: The thread connection between the three parts of the device

That mechanism, although it seemed simple, it was rejected as well. The main reason was the limitations of the 3D-printer in combination with the resin properties, in the context that even between the same resin parts the threads couldn't connect as they wouldn't fit. The threads would need much micro tuning in order to work and that would be very time-consuming taking into account the time taken for the different prototypes to be printed and tested in order to make the mechanism work. On top of that, the same process would need to be done for the two parts with the different resins, meaning more prototypes and time consumption. Besides, a possible replacement of the ABS like resin with another with similar properties for economic or quality reasons would mean that all this work would need to be done again, something very costly and time ineffective. Finally, the circular motion when connecting or disconnecting the parts is not considered ergonomic.

The third solution, which was the one approved, was the push fit mechanism. This solution is depicted in figures 2.9 and 2.10.

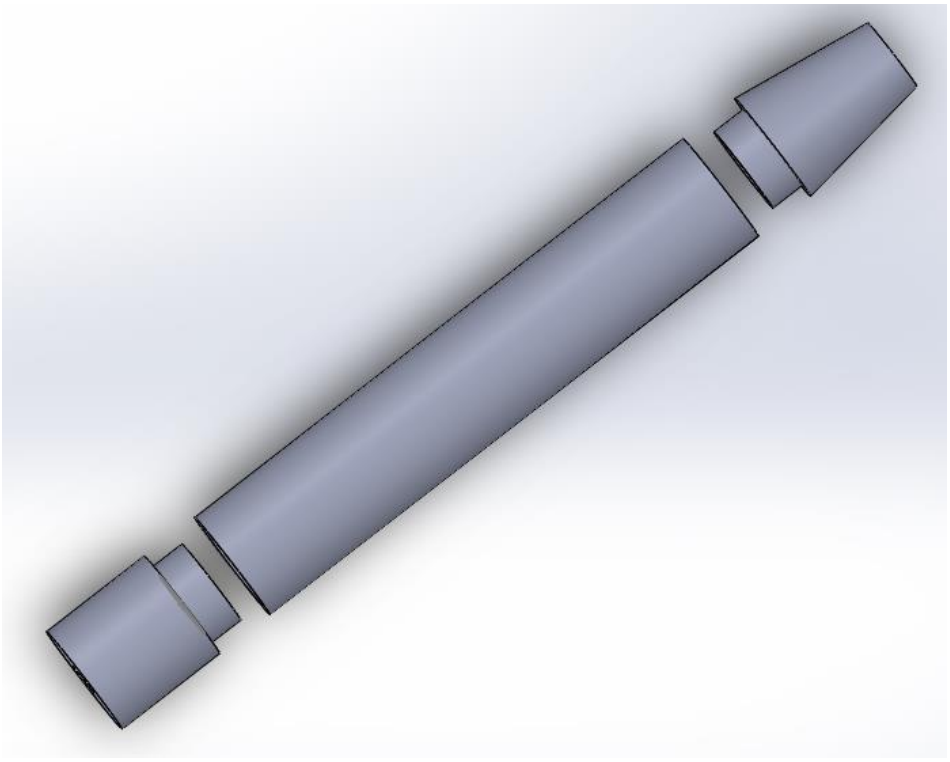

Figure 2.9: The third solution with the push fit mechanism for the device

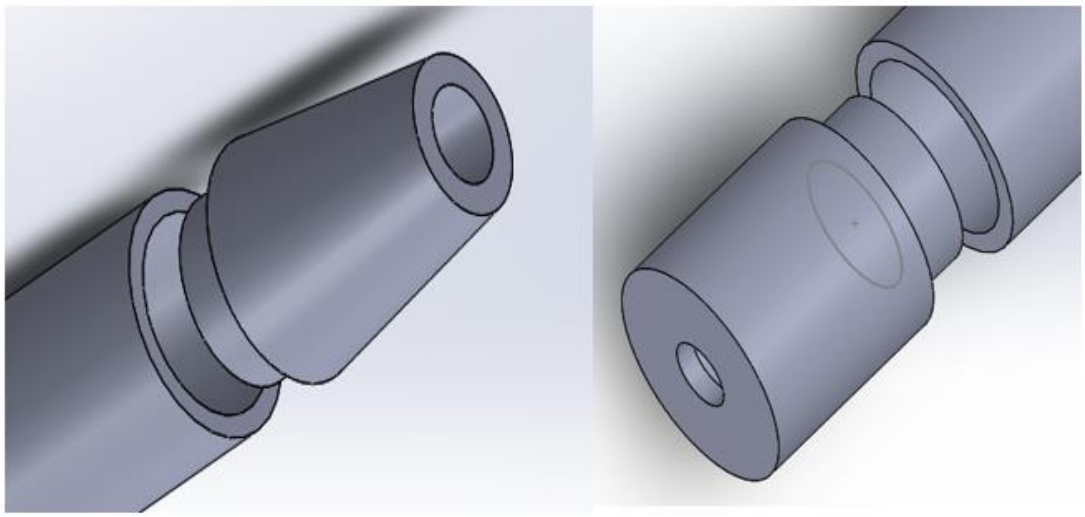

Figure 2.10: The push fit mechanism between the three parts of the device

This type of mechanism is based on a small difference between the connecting diameters of the three parts of the parts. One part has a slightly bigger diameter and the other one has a slightly smaller diameter, with the difference being of the order of hundredths of millimeters. Push fit mechanism has some important advantages. First of all, it can be 3D-printed easily and without significant accuracy problems. It also doesn't present any problems regarding the connection between parts from different resins despite their different properties. The movement in order to connect or disconnect the parts of the device is linear and simple to do. This movement is also considered ergonomic. As a result, the mechanism is both user friendly and simple.

2.2.3 Final solution

After carefully analyzing each of the previously discussed alternatives, the final design of the device was established. It consists of three parts and the parts are connected with the push fit mechanism. Ergonomic, simple to use and to 3D print, while being non-invasive. Furthermore, it's portable and can be in small packaging. The extra safety measure is positioned on the top part. Specifically, the diameter of the hole in the top part is much smaller than the diameter of the paper filter, in combination with the conical shape of the tip. Hence, there is no danger of swallowing the paper filter if, for some reason, the user inhales instead of exhales as instructed. This scenario could be considered rare since the instruction is to simply exhale on a single continuous exhalation for a specific number of times, but it is better to have a measure for that. The device is depicted in the following figures 2.11 and 2.12.

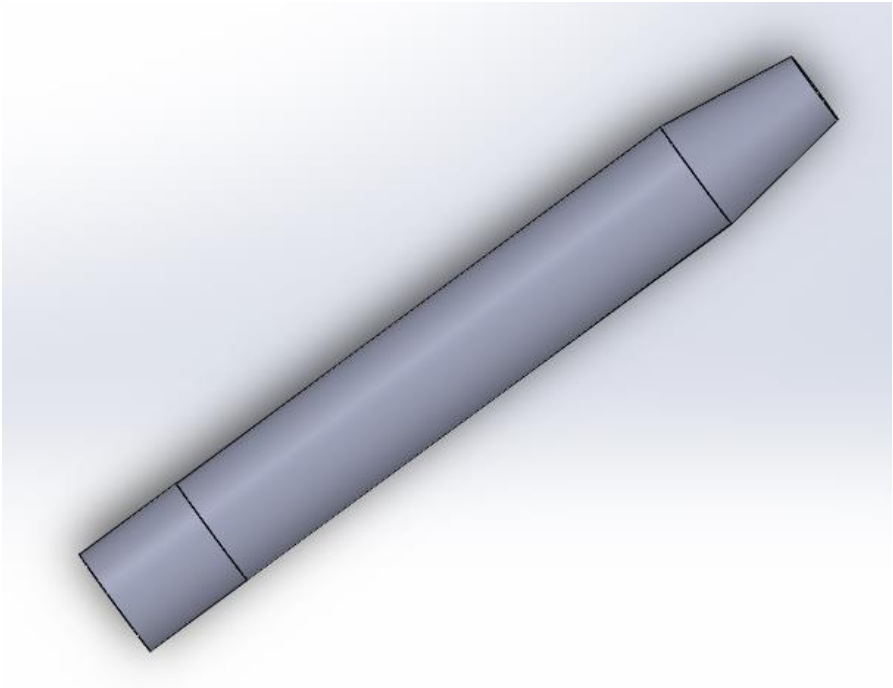

Figure 2.11: The assembly of the final solution for the design of the device

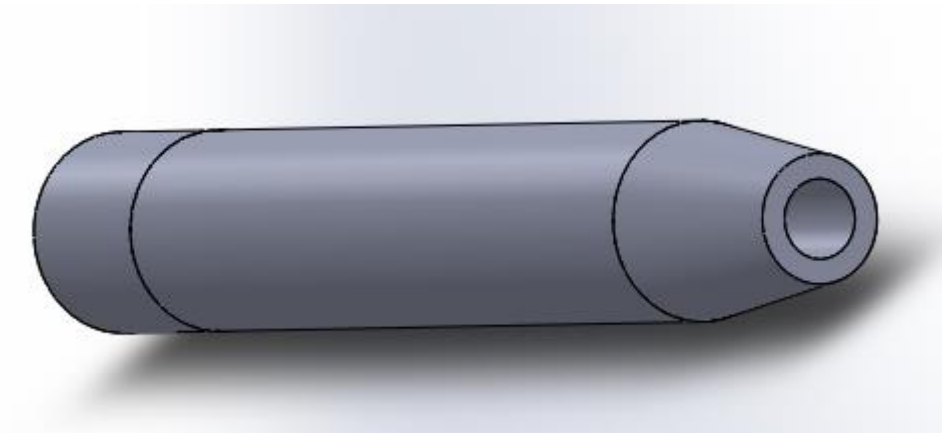

Figure 2.12: The assembly of the device

2.3 Prototyping

Many alternative solutions were prototyped with the aim of finding the most feasible and to guarantee that the user will find it pleasant and simple to use.

The second solution was quickly rejected after a few prototypes due to the thread issues that were mentioned before. After that, the prototypes were focused on the third solution with the push fit mechanism. The objectives of these prototypes were

many. At first, the top part was 3D-printed with the medical resin. The objective was to ensure that the surface of this part is smooth and doesn't have any roughness that could cause discomfort. After ensuring the surface quality of the medical resin, the three parts of the device were 3D-printed in the ABS like resin. In the figures 2.13 we can see those parts.

Figure 2.13: Prototypes from the three parts of the product

The purpose behind this was to first check the surface quality of the other parts and to be sure that they are smooth and suitable for the user to hold. Secondly, it was important to find the right tolerance between the diameters of the parts for the push fit, in order for them to connect easily and have a steady hold. Figure 2.14 shows other prototypes that were used to find the right tolerance.

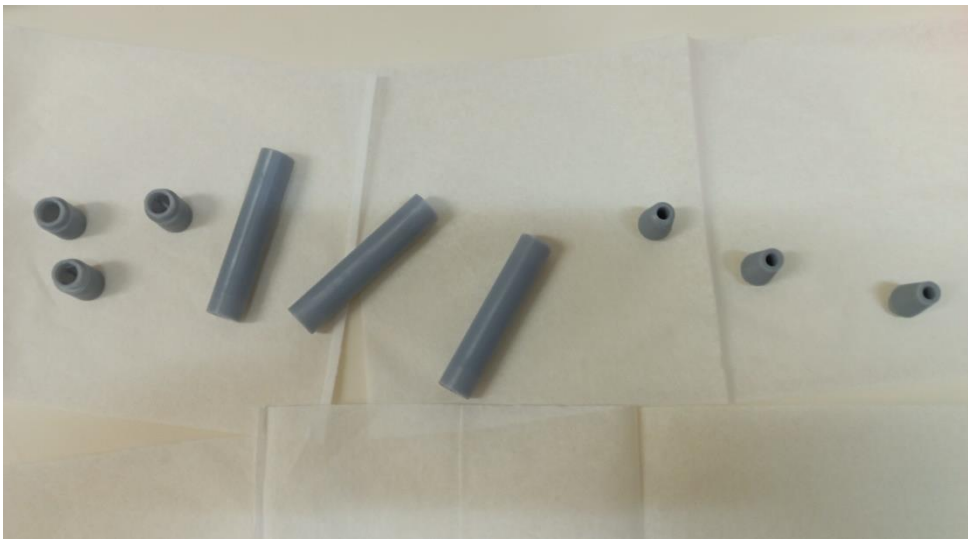

Figure 2.14: Prototypes used for the right tolerance of the push fit mechanism

The 3D-printed bottom parts were also tested to make sure that the paper filter would be put in and taken off with ease.

A variance of the top part was also 3D-printed in both resins as an alternative solution that could be more user friendly because of its smoothened edge. The different top part is presented in the figure 2.15.

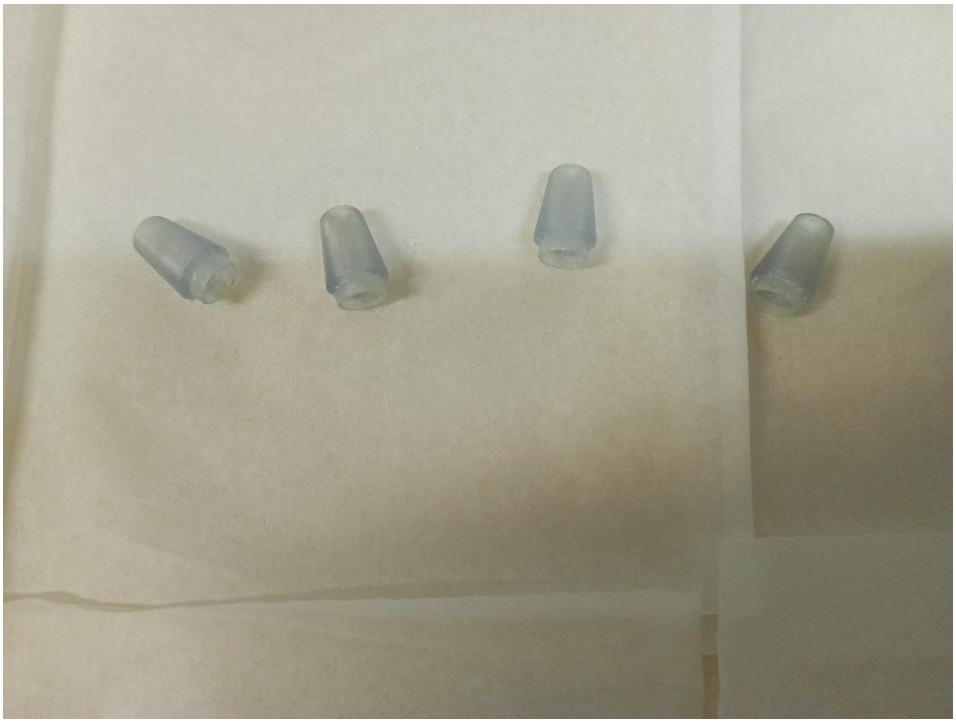

Figure 2.15: Different 3D-printed top parts/tips

The difference between them is more visible in the next figure 2.16.

Figure 2.16: Different edges for the top part of the device

The different top part didn’t have a major difference in terms of user friendliness but rather served a different aesthetic, so it was discontinued.

The final design was very influenced by the prototyping, since the prototypes helped to get a better understanding of the needs of the user and what difficulties the user could face like with the thread or the snap fit.

3. Experiments and feasibility of the device

After the final design was established and a prototype had been created, experiments needed to be done in order to check the device's feasibility and if all the specifications were being met. Before the experiments started, copies of the parts of the device were printed in order to have some spares. Moreover, the sterilization of those parts was necessary. The parts printed with medical resin (top parts) were sterilized in an autoclavable machine which made them odor less and ready to use. It is important to mention that the medical resin is certified and the user can keep it in his/her mouth for 30 minutes, time that is more than enough for breath sampling. The parts with the ABS like resin were sterilized using 70% ethanol and a UV lamp. Then the parts were ready for use. The last step before starting the experiments was to cut the paper filters from a plain conjugate pad. The paper filters had a diameter of 6mm and were cut using a biopsy punch of the same diameter. The filters are depicted in figure 3.1.

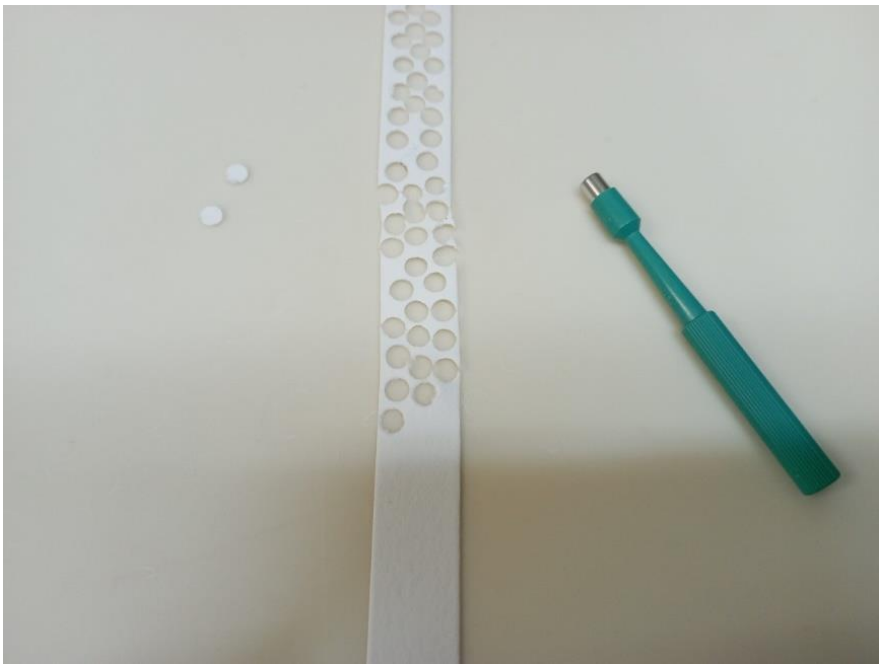

Figure 3.1: The cutting of the paper filters for device

The experimental setup is presented in the figure 3.2.

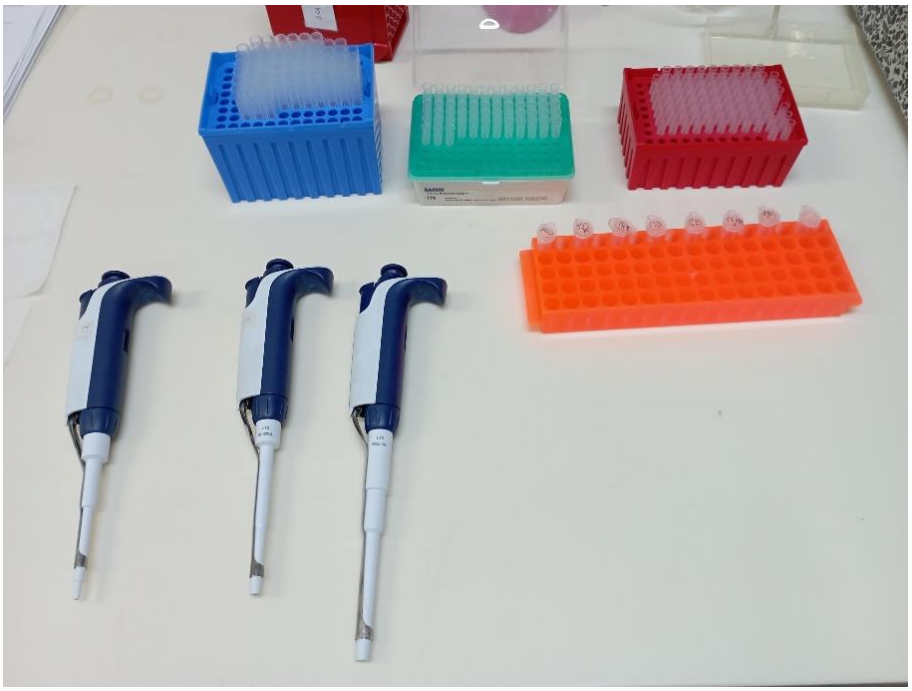

Figure 3.2: The experimental setup used for the experiments

3.1 Protein detection by observing color change

In the first experiments, the aim was to detect protein collected in the paper filter by observing color change and to determine the external factors that can have an impact on the results. They served as a first indication of whether there is protein presence or not.

3.1.1 Experimental procedures

The procedure for the protein detection had some simple steps:

1) Place the paper filter at the end of the bottom part and assemble the device.
2) Take a deep breath and blow through the device in a single continuous exhalation. Repeat for 5 exhalations in total.
3) Take the paper filter out with a tong and place it inside a well of a 96-well plate.
4) Add 200 μl of PBS in the same well with the use of a suitable pipette and leave the sample for 30 minutes in room temperature.
5) Place a paper filter inside a tube with saliva and leave it for 30 seconds.

6) Take the paper filter with the saliva and place it inside a well.
7) Add 200 μl of PBS in the same well with the use of a suitable pipette and leave the sample for 30 minutes in room temperature. This sample is the positive control.
8) Add 100 μl of PBS to have a blank – negative control sample.
9) Take 100 μl from the two wells containing the paper filters using different tips each time and place each on a different well.
10) Add 100 μl of the working solution of the BCA Protein Assay kit to each of the three wells. The working solution is created by mixing BCA Reagent A and BCA Reagent B at a 100:1 ratio.
11) Incubate in 37 ℃ for 30 minutes.
12) Cool plate at room temperature.

The experiments were not restricted to only that exact procedure but other factors were examined. As it was mentioned before, a standardized breath sampling procedure needed to be established in order for the results to be repeatable and due to the fact that the procedure itself has a significant impact on the results. At first, the most suitable method for breath sampling was inspected. There were three different methods that were considered and tested using the procedure stated above. The first one was using normal – light breathing and exhaling through the device 5 and 10 times. The same method was repeated but for 20 exhalations. Afterwards, the “drinking straw” method was tested for 5 and 10 exhalations. Finally, the “flute” method was used for 5 exhaled breaths. The “flute” method was picked as the most suitable for reasons that will be explained in the next section. After the method was picked, the external factors needed to be examined. More specifically, there was the need to make sure that the paper filters alone didn’t contain an amount of protein that would make them unsuitable to be used and undermine the experiments. For that reason, the same procedure was followed, but this time an unused paper filter was used instead. In addition, it was essential to test if the contact between the paper filter and the resin of the bottom part could contaminate them and have an impact on the results. In order to test that, a paper filter was rubbed on a spare bottom part made of ABS like resin, and then the same procedure was followed using that one instead of the one used for breath sampling. The results from these experiments will be discussed in the next section.

3.1.2 Results

A result from the first experiments is shown in the following figure 3.3.

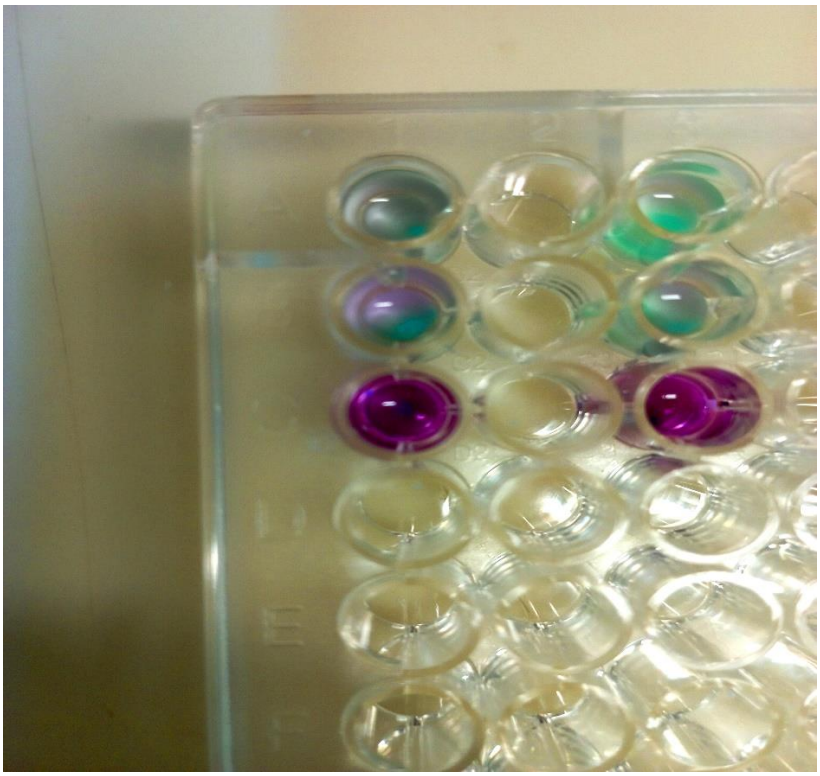

Figure 3.3: A 96 well plate from the light breath experiments

In the first row of the plate, the two wells contained plain PBS and served as the negative control. In the second row, the two wells contained samples after 20 light exhaled breaths (left) and 10 light exhaled breaths (right). The third row is from the saliva dipped samples that worked as the positive control. By observing the color difference, it was evident that there was protein presence in the samples that were used from light breaths.

From the results it was clear that each of the three sampling methods worked. The first one with the light exhaling was rejected because even though it could be considered the most user-friendly of the three, the paper filter wasn't collecting a significant concentration of protein, even though the light exhalation was repeated for more exhaled breaths than the others. The "drinking straw" method, although it gave good results, was rejected mostly due to the fact that there isn't a standard and sure way to know how long the partial exhaled breath should be or how much air should be exhaled. Furthermore, since the device is set to be used by people from different age groups, it would be difficult for certain users to cooperate with that type of method. The "flute" method was picked because it gave higher protein

concentration with fewer repeats and because it was the method that most users would be able to follow without difficulty.

Regarding the external factors, no color change was noticed in both the blank paper filter and the one that was rubbed on the bottom part. Therefore, the experiments could continue without their results being undermined. The results can be seen in figure 3.4.

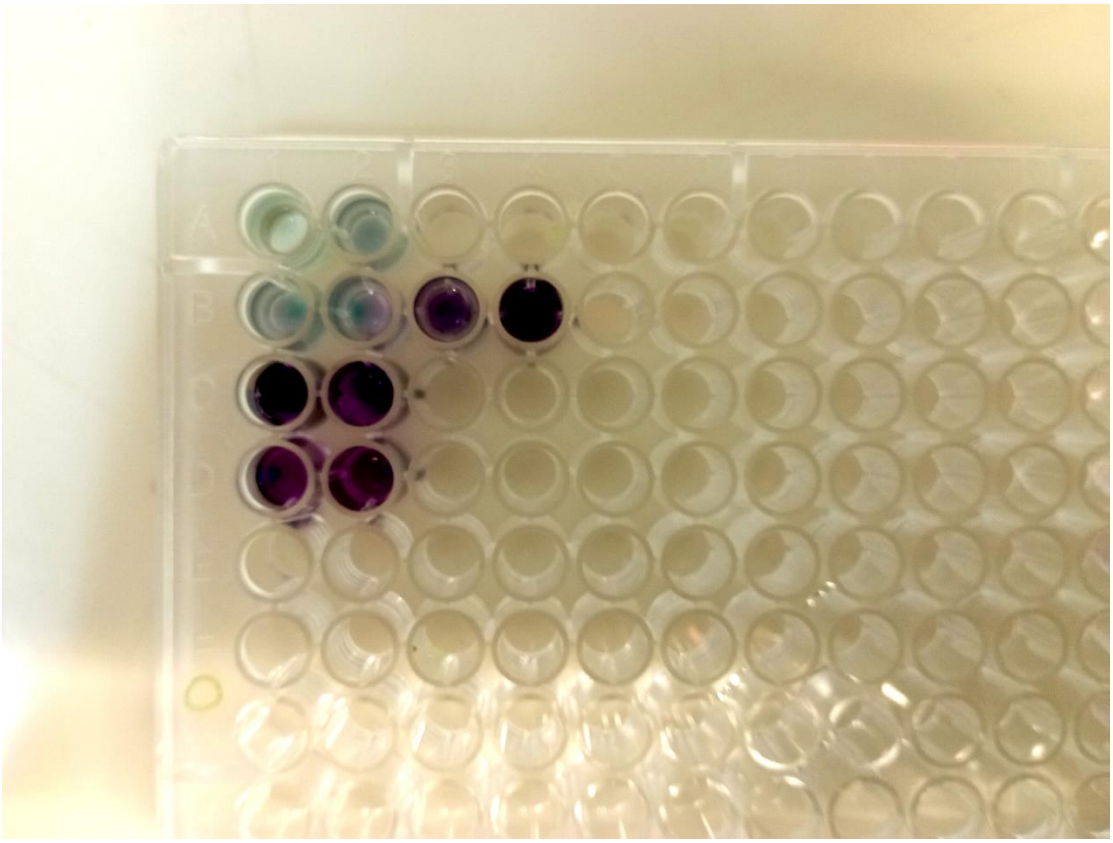

Figure 3.4: A 96 well plate from the experiments for different ways of exhaling and the external factors

This plate was an example of the experiments examining the external factors. The well A1 (top left) contained a sample from an unused filter and the well A2 contained a sample from a paper filter rubbed on the resin. It was clear that there was no color change in them. In the second row, starting from left to right, the first sample contained 10 light exhalations, the second contained 20 light exhalations, the third contained 10 exhalations using the “flute” method and the last one was saliva dipped. The third row contained samples with 20 exhalations each after using the “flute” method and the fourth row contained samples with 20 exhaled breaths each after using the “drinking straw” method. It is important to note that the color of the samples is to some extent highlighted due to the large quantity of the working solution. That is mentioned because in the next figures the colors might seem slightly lighter. Nonetheless, the more intense colors were crucial in order to have a first indication of protein presence and because the first experiments were more qualitative than the next ones.

3.2 Protein detection using the BCA analysis for different numbers of exhaled breaths

Since the presence of protein was proved, the next step was to try to perform a BCA analysis to find the protein concentration in the samples for different numbers of exhaled breaths. The reason behind this was to determine what number of breaths was suitable and should be set in order to have feasibility.

3.2.1 Experimental procedures

The procedure is being described in the BCA Procedure that is in the Annex of the thesis. The procedure was followed for 1,2,3,4 and 5 exhaled breaths in each sample. Then, it was decided to increase the number of exhaled breaths to 7,8,9 and 10 times in each sample.

A slightly different procedure was also being used for the experiments and is described in the Second BCA Procedure in the Annex. The objective behind the slightly different procedure was to determine if a denser sample could give better results. This procedure was followed for 7,8,9 and 10 exhalations. Then, once again, the exhaled breaths were increased to 5,10,15 and 20 in each sample.

It has to be mentioned that because of the results, there was no point in having a positive control sample. Nonetheless, the concentration of a sample that followed the procedure with the saliva mentioned earlier was measured. Likewise, the absorbance of both an unused paper filter and a paper filter rubbed with a resin part were measured, in order to have both qualitative and quantitative evidence that they didn't have an impact.

3.2.2 Results

At first, the results for 1,2,3,4 and 5 exhaled breaths in each sample were giving a very low protein concentration in each sample, albeit a higher concentration than the blank well with the plain PBS. Even with the 7,8,9, and 10 exhalations, the protein concentration remained low. The denser samples gave higher concentrations than the previous samples but the problem was that they couldn't fit in the standard curve of the BCA. Also, it was clear that after a point, the samples didn't have much difference when it came to protein concentration, even in the denser form. This is something that can be expected since the concentrations are low and a linear correlation between exhaled breaths and concentration would be difficult to depict. The procedure with the 5,10,15 and 20 exhalations was actually more successful since each sample had a higher concentration than the blank one and in most cases the sample with the higher number of exhalations had a higher protein

concentration. A good example from the results with the denser form is presented in the next figures 3.5, 3.6 and 3.7.

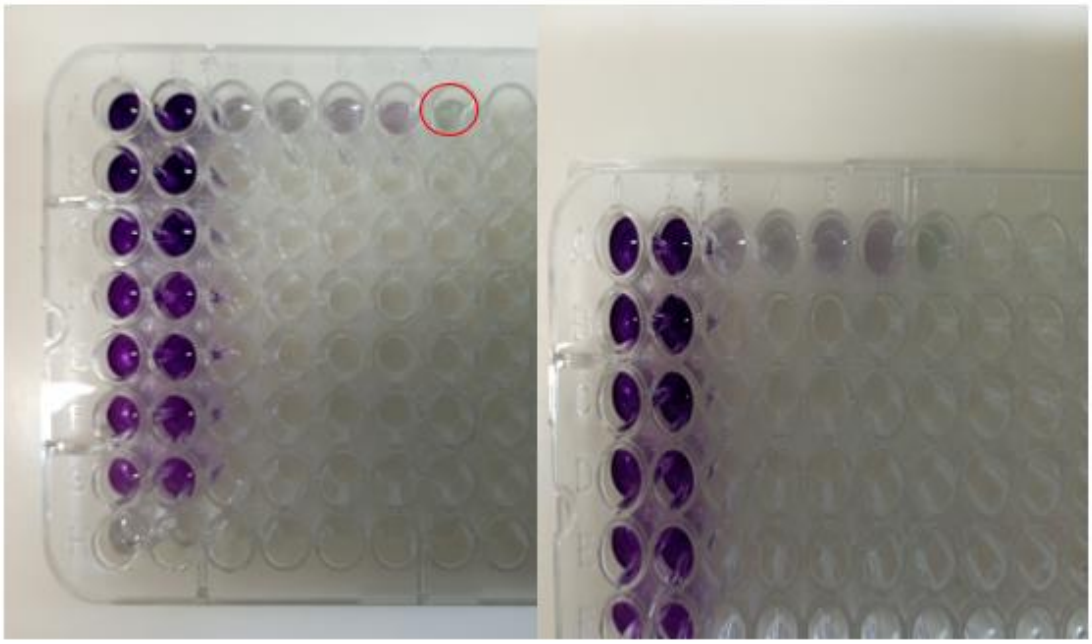

Figure 3.5: A 96 well plate from the experiments using BCA analysis for different numbers of exhalations

	1	2	3	4	5	6
A	2000	2000	5 exhalations	10 exhalations	15 exhalations	20 exhalations
B	1500	1500				
C	1000	1000				
D	750	750				
E	500	500				
F	250	250				
G	125	125				
H	0	0				

Figure 3.6: The plate layout of the 96 well plate in the figure 3.5

Concentration of BSA (μg/ml)	Mean absorbance	Samples	Absorbance		
2000	2.7375	5 exhalations	0.235		
1500	2.66	10 exhalations	0.200		
1000	2.123	15 exhalations	0.260		
750	1.625	20 exhalations	0.266		
500	1.5295				
250	1.1335				
125	0.901				
0	0.1325				

Figure 3.7: The measured absorbance of the 96 well plate of figure 3.5

From the figures above, the presence of protein was obvious both qualitatively because of the color and quantitatively from the absorbance difference between the samples and the blank. It is also clear that those samples cannot fit in the standard curve. The sample on the A7 well, marked with the red circle, is a sample from an unused paper filter whose absorbance was similar to the blank (0.140).

Even the new numbers couldn't fit into the standard curve, so it was clear that the standard curve should depict lower concentrations than the standard ones. At the same time, an important observation was made. After leaving the samples for 48 hours instead of the usual 30 minutes, their absorbance was significantly higher. This led to valuable changes in the protocol with both different numbers of exhalations and different time needed for the sample to be left after the working solution is added. The vast difference is depicted in figure 3.8.

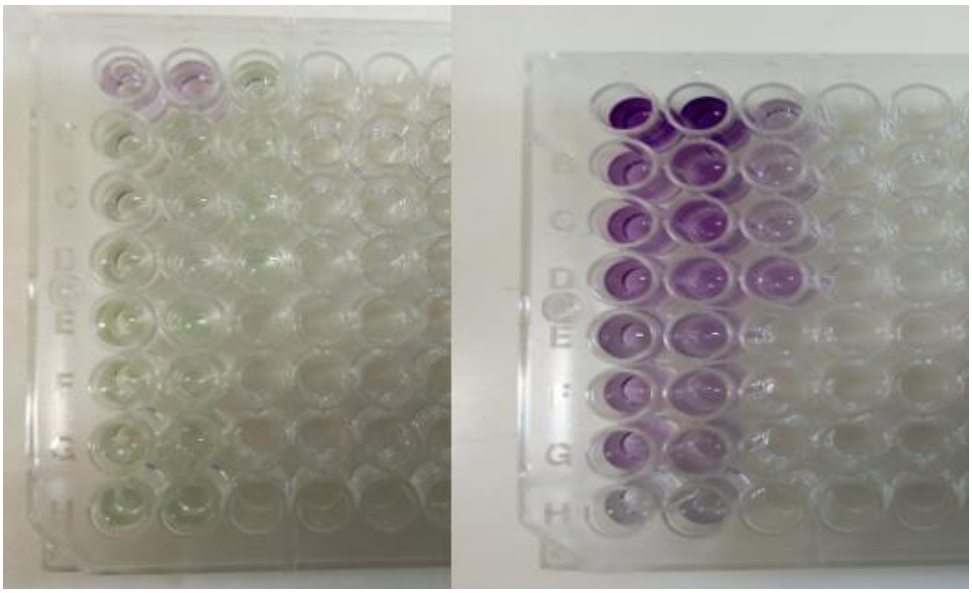

Figure 3.8: A 96 well plate from the experiments before and after 48 hours

3.3. Protein detection using BCA analysis and quantification on protein concentration

Since the number of exhalations that were suitable was established and there were the necessary changes in the procedure, in the next experiments the BCA analysis was performed again using the new and improved protocol. The objective this time was to ensure the feasibility of the test and try to quantify the concentration of the samples even approximately.

3.3.1 Experimental procedures

The whole new procedure is being described Updated BCA Procedure that is located in the Annex of the thesis.

3.3.2 Results

With the new procedure, the results were encouraging. First, the feasibility of the test was proven because in each case the protein concentration in every sample was significantly higher than the blank and in some cases the concentration was even measured and was considered adequate. One result from these experiments is depicted in the figures 3.9, 3.10 and 3.11.

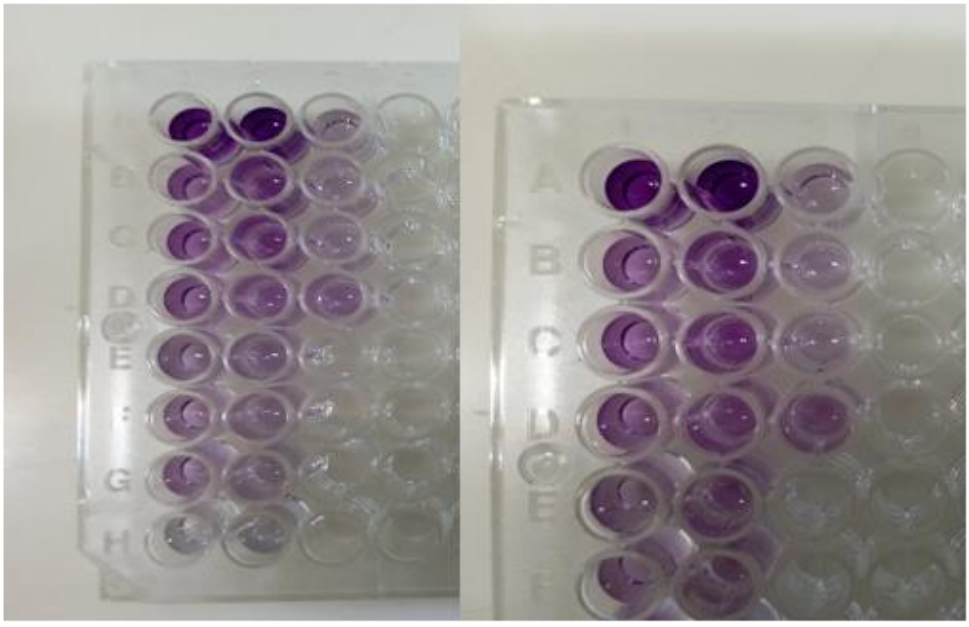

Figure 3.9: A 96 well plate from the experiments using the new protocol for the BCA analysis

	1	2	3	4	5	6
A	500	500	5 exhalations			
B	250	250	10 exhalations			
C	125	125	15 exhalations			
D	62,5	62,5	20 exhalations			
E	31,25	31,25				
F	15,625	15,625				
G	7,8125	7,8125				
H	0	0				

Figure 3.10: The plate layout of the 96 well plate pictured of the figure 3.9

Concentration of BSA (µg/ml)	Mean absorbance	Samples	Absorbance		
500	1,488	5 exhalations	0,236		
250	0,6035	10 exhalations	0,236		
125	0,589	15 exhalations	0,231		
62,5	0,4705	20 exhalations	0,367		
31,25	0,3825				
15,625	0,3265				
7,8125	0,3025				
0	0.167				

Figure 3.11: The measured absorbance of the 96 well plate of figure 3.9

From these results, the presence of protein was evident since all the samples had significantly higher absorbance than the blank sample. Additionally, although the first three samples couldn't fit in the BSA curve, the last could and its protein concentration was measured at 47,48 µg/ml.

Another result from the experiments is presented in the figures 3.12, 3.13 and 3.14.

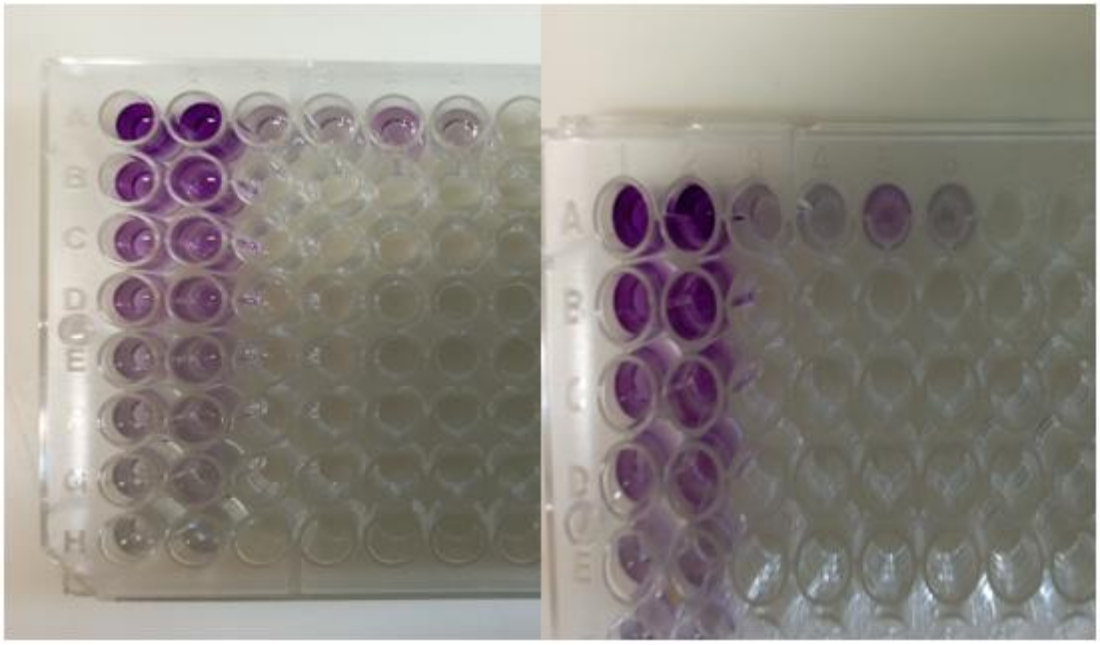

Figure 3.12: A different 96 well plate from the experiments using the new protocol for the BCA analysis

	1	2	3	4	5	6
A	500	500	5 exhalations	10 exhalations	15 exhalations	20 exhalations
B	250	250				
C	125	125				
D	62,5	62,5				
E	31,25	31,25				
F	15,625	15,625				
G	7,8125	7,8125				
H	0	0				

Figure 3.13: The plate layout of the 96 well plate of the figure 3.12

Concentration of BSA (μg/ml)	Mean absorbance	Samples	Absorbance		
500	1,306	5 exhalations	0,209		
250	0,807	10 exhalations	0.201		
125	0,537	15 exhalations	0.34		
62,5	0,4255				
31,25	0,2755				
15,625	0,231				
7,8125	0,1825				
0	0,128				

Figure 3.14: The measured absorbance of the 96 well plate of the figure 3.12

In these results, the presence of protein was yet again evident, since all the samples had significantly higher absorbance than the blank sample. Furthermore, all four samples could fit in the curve and their protein concentration was measured. The first sample had a protein concentration of 8,75 μg/ml. The second sample had 5,67 μg/ml and the third one had 59,13 μg/ml. The last sample had low absorbance, which was an exception from the majority of the results where it always had the higher absorbance of the samples, but it was decided for those results to be presented because of the good absorbance and protein concentration of the other three samples.

4. Discussion and conclusions

4.1 Current limitations

Even though the device has some exceptional characteristics that were examined earlier, it is not without a flaw. There are some limitations that should be mentioned and solved in order for the device to become better and be used in other applications.

First of all, the paper filter could be better. The current one used is made out of a plain conjugate pad, which makes it pretty dense. Although the density helps in the collection of compounds and proteins from the exhaled air, at the same time, the air passes with some resistance. That resistance is not uncomfortable during the sampling process when the exhalation is repeated five times, but after that, it starts to potentially get tiring. The number of exhalations itself can be tiring after a certain point, but still, a more lightweight and improved filter can provide a better experience for the user with the sampling method and allow for more repeated exhaled breaths.

Moreover, if the device's parts continue to be 3D-printed, it would advisable that the product should be tested for toxicity before the start of any clinical trial. The resin used for the top part (biomedical resin type 1) has certifications for both ISO 10993-5:2009 and ISO 10993-10:2021. The first ISO describes test methods to assess the in vitro cytotoxicity of medical devices and the second ISO specifies the procedure for the assessment of medical devices and their constituent materials with regard to their potential to induce skin sensitization. The other two parts are made from ABS like resin which is not considered toxic, but even so, testing the device should be considered for the protection of people participating in those clinical trials.

Lastly, the manufacturing process could be better. Considering a high demand for the product, the 3D-printing is not the best option for manufacturing, since there are other procedures that can produce the parts at a faster time and rate. The surface quality can be considered good, but there are limitations on the connecting mechanism of the device. The device is intended to be ready to use right out of the package, but still, the bottom part will need to be taken off in order to take the paper filter for analysis. Hence, there can be a preference from the user for one mechanism over the other.

4.2 Future development

4.2.1 Technical development

The following technical approaches should be taken into account if the product is going to be developed for the market. As a start, there needs to be a different manufacturing method given the possibly high demand for the device, as it was explained earlier. Accordingly, a cast for injection molding should be created for the product. This process is suitable for mass production while giving better accuracy and quality. It also allows the parts to connect with both the push-fit mechanism and a thread, meaning more options for the user. As a result, the device will have a more steady and better connection between its parts with both the push-fit mechanism and the thread, making it better for the user. While using this method, it can be considered to use plastic that has a color or colors similar to the ones that color crayons have, which will increase the marketability of the product and make it more appealing especially for children, provided that the plastic used is suitable and safe for the user. It can also be expected that the manufacturing costs will be lower by using this process, because medical resin is expensive.

Along with this, a better paper filter can help to make the sampling process better. Filters from different materials could be considered as long as they don't affect the experimental results. It should be noted that this is not as crucial as the different process for manufacturing. The reason behind this is that from the results, even with five exhalations, the sample had a significantly higher protein concentration than the blank sample, so five exhalations can be considered adequate. The discomfort for the user during those five repeats is very low as it was explained earlier.

4.2.2 Development into a new lateral flow test

The big advantage of this device is that it can become a part of a two-step lateral flow test for various protein biomarkers. During the experiments, it was proved that there is protein in an exhaled breath and it was approximately measured. The paper filter was essentially collecting this protein. The device can be the first step of a lateral flow test, as the sampling method. More specifically, after the user has exhaled 5 times through the device, the paper filter can be taken off and be placed in a well using a tong provided in the package. The well doesn't need to be a part of the whole 96 well plate, but it can be a single well packed with the lateral flow test. Then, the user will add the PBS inside that well and will leave the sample for 30 minutes. The PBS can be inside a small tube in the package. After the 30 minutes, the user will use a small plastic pipette to take the liquid and then let it flow along the test, passing through the conjugate pad into the nitrocellulose membrane and then onto the absorbent pad. The test can have the same design of the lateral flow tests

used for COVID-19. This test can also be done in a simpler way using a tube-like pipette like the ones used for COVID-19 self-tests. This type of pipette is presented and marker with a red ellipse in the following figure 4.1.

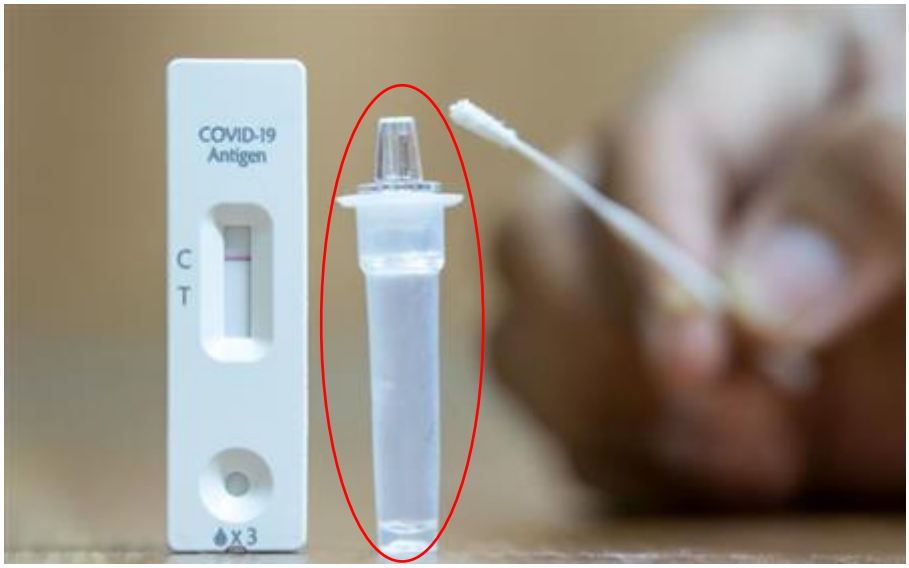

Figure 4.1: The tube-like pipette used in some COVID-19 self-tests [16]

The tube-like pipette will already contain the PBS and the user will just drop the paper filter inside using a tong. Then, after 30 minutes have passed, the user will turn the pipette upside down and let the liquid flow through the test. If the paper filter makes it difficult for a few drops to pass through, although unlikely, a second clean tong can be provided to take out the filter and then turn the pipette upside down.

The public is familiar with the process of self-tests, so it won't be difficult for them to follow these steps and use the test. A self-test kit is depicted in the figure 4.2.

Figure 4.2: A COVID-19 self-test [17]

There is research that confirms the presence of proteins in exhaled air and has identified some of the proteins [18]. Proteins have also been identified as biomarkers for diseases [19|20].

The lateral flow tests can have those proteins as labels and detect various diseases while being completely non-invasive and user-friendly. They also won't need analysis in a specialized laboratory and the user can have the results fast, revolutionizing diagnostics.

4.2.3 From prototype to end product

It is obvious that developing the prototype into an end product is a multifaceted process which requires the examination of many factors that cannot be overlooked. First of all, a business plan needs to be created and analyzed. This involves market research, financial planning and other complex steps. In order for the business plan to be fully implemented, it necessitates specialized personnel that will examine and analyze all these factors that surround the product.

An essential factor is the biocompatibility and safety of the device. The device needs to comply with the US-FDA and EU-MDR regulations for type I medical devices. Also, in the case that the manufacturing process is injection molding, the plastic that will be used needs to be biocompatible, non-toxic and safe to use. The verification and validation of the device are also important.

Regarding the lateral flow tests, they require a lot of testing. Their sensitivity and specificity need to be measured and large-scale clinical trials need to be conducted in order to test if the biomarker can be detected and the tests work properly. It is evident that a team of engineers needs to work on this type of tests and the participation of volunteers will be needed. Additionally, it needs to be decided whether the lateral flow tests will be bought ready to use from a company or equipment will be bought with the aim of producing them.

The logistics is another aspect that should not be overlooked. The product should be packaged alone or with the lateral flow test together, preferably in small packages. It needs to be decided where the product will be distributed and to make a deal with the transportation company to ensure low transportation costs for the product, so as to have a marketable product.

Lastly, it is crucial to protect the intellectual property. For that reason, a patent attorney should be hired to conduct the proper research for the Intellectual Property and write a proposal to the European Patent Office for the product. The patent could focus on the sampling method and the lateral flow tests supposing they work as intended.

4.3 Conclusions

As a conclusion, this product has the potential to revolutionize the diagnostics industry. It is non-invasive, simple and user-friendly. In combination with the lateral flow tests, it can replace existing uncomfortable sampling practices and diagnostic procedures and become the new standard. It covers the need of the public to monitor its health, making it a desirable product.

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6. Annex

1. BCA Procedure
2. Second BCA Procedure
3. Updated BCA Procedure
4. Mechanical drawings of the device