Development and Optimization of a DNA extraction protocol through microscale solid phase extraction (μSPE) and microfluidics for food analysis applications

Joana Raquel Rodrigues Carvalho

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Supervisors: Professor Susana Isabel Pinheiro Cardoso de Freitas

and Doctor Marta Prado Rodríguez

Examination Committee

Chairperson: Professor Gabriel António Amaro Monteiro

Supervisor: Doctor Marta Prado Rodríguez

Member of the Committee: Professor Luís Joaquim Pina da Fonseca

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ABSTRACT

DNA based analytical procedures often require big specialized equipment, costly reagents, highly trained personnel and large sample sizes, limiting its applicability as routine analysis in the food industry. The work developed for this project is part of a larger research effort to develop a portable and integrated DNA analysis device for in situ applications in the food industry sector, where is intended to include all the steps of DNA analysis in a single device. There is a growing interest in combining emerging technologies at microscale with well-established molecular biology procedures in order to improve the steps of DNA analysis: Extraction, Amplification and Detection. This project is focused on the first step of DNA analysis and its main goal is the development and optimization of a DNA extraction protocol through microscale solid phase extraction (μSPE) and microfluidics for food analysis applications. Therefore, washable and reusable systems containing commercial disposable silica membranes were used to optimize each step of DNA extraction, namely binding, washing and elution, by testing a set of protocols that could be integrated onto micro Total Analysis Systems (μTAS). In each step, samples were collected for quantification on a micro-volume fluorospectrometer to compare and select protocols according with their DNA yield. One of the tested protocols, involving a non-chaotropic binding buffer, an ethanol free washing step and a short incubation on buffer TE for DNA elution, achieved the best results regarding DNA yield and feasibility of the procedure for a microfluidic setting.

Key words: DNA extraction, Food analysis, Microscale solid phase extraction, Microfluidics.

RESUMO

A análise de ADN exige equipamentos especializados de grande dimensão, reagentes caros, pessoal altamente treinado e grandes volumes de amostra, limitando a sua aplicabilidade como análise de rotina na indústria alimentar. O trabalho desenvolvido nesta dissertação faz parte de um projeto mais alargado para o desenvolvimento de um dispositivo portátil de análise de ADN destinado a aplicações in situ na indústria alimentar. Tem havido um interesse crescente na combinação de tecnologias de microescala com procedimentos bem estabelecidos de biologia molecular para melhorar as etapas da análise de ADN: extração, amplificação e eluição. Este trabalho foca-se na primeira fase desta análise e o principal objetivo é o desenvolvimento e otimização de um protocolo de extração de ADN através de técnicas de microextração em fase sólida (μSPE) e microfluídica para análise de alimentos. Assim, foram utilizados sistemas laváveis e reutilizáveis contendo membranas descartáveis de sílica para otimizar cada etapa da extração, nomeadamente os passos de ligação, lavagem e eluição, testando um conjunto de protocolos adequados para integração em microssistemas de análise total (μTAS). Em cada passo, recolheram-se amostras para quantificação com um espectrofluorímetro de micro-volume de modo a comparar e selecionar os protocolos com melhor rendimento de extração. Um dos protocolos testados, usando um tampão de ligação com sais não-caotrópicos, um passo de lavagem livre de etanol e uma incubação curta com tampão TE para a eluição do ADN, demonstrou ter os melhores resultados relativamente ao rendimento de extração e à viabilidade do procedimento para integração num sistema de microfluídica.

Palavras-chave: Extração de ADN, Análise Alimentar, Microextração de fase sólida, Microfluidica.
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<th>Description</th>
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<tr>
<td>A</td>
<td>Absorbance</td>
</tr>
<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>BB1</td>
<td>Binding buffer 1</td>
</tr>
<tr>
<td>BB2</td>
<td>Binding buffer 2</td>
</tr>
<tr>
<td>BB3</td>
<td>Binding buffer 3</td>
</tr>
<tr>
<td>BB4</td>
<td>Binding buffer 4</td>
</tr>
<tr>
<td>BIP</td>
<td>Backward Inner Primer</td>
</tr>
<tr>
<td>b</td>
<td>Path length of the sample [cm]</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSE</td>
<td>Bovine spongiform encephalopathy</td>
</tr>
<tr>
<td>C</td>
<td>Concentration of the compound in solution [M]</td>
</tr>
<tr>
<td>C</td>
<td>Cytosine</td>
</tr>
<tr>
<td>CJD</td>
<td>Creutzfeldt-Jakob disease</td>
</tr>
<tr>
<td>( C_t )</td>
<td>Threshold cycle</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyltrimethyl ammonium bromide</td>
</tr>
<tr>
<td>dATP</td>
<td>Deoxyadenosine triphosphates</td>
</tr>
<tr>
<td>dCTP</td>
<td>Deoxycytidine triphosphates</td>
</tr>
<tr>
<td>dGTP</td>
<td>Deoxycytidine triphosphates</td>
</tr>
<tr>
<td>DI</td>
<td>Deionized</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphates</td>
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<tr>
<td>dsDNA</td>
<td>Double-stranded DNA</td>
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<td>dTTP</td>
<td>Deoxythymidine triphosphates</td>
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<td>E1</td>
<td>Elution buffer 1</td>
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<tr>
<td>E2</td>
<td>Elution Buffer 2</td>
</tr>
<tr>
<td>EC</td>
<td>European Commission</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>FIP</td>
<td>Forward Inner Primer</td>
</tr>
<tr>
<td>G</td>
<td>Guanine</td>
</tr>
<tr>
<td>GM</td>
<td>Genetically modified</td>
</tr>
<tr>
<td>GMO</td>
<td>Genetically modified organism</td>
</tr>
<tr>
<td>IgE</td>
<td>Immunoglobulin E</td>
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<tr>
<td>LAMP</td>
<td>Loop-mediated isothermal amplification</td>
</tr>
<tr>
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<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LOC</td>
<td>Lab-on-a-chip</td>
</tr>
<tr>
<td>MEMS</td>
<td>Microelectromechanical systems</td>
</tr>
<tr>
<td>NASBA</td>
<td>Nucleic acid sequence based amplification</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>PCB</td>
<td>Polychlorinated biphenyl</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>PDO</td>
<td>Protected Designation of Origin</td>
</tr>
<tr>
<td>PGI</td>
<td>Protected Geographical Indication</td>
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<tr>
<td>PGS</td>
<td>Protected geographical status</td>
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<td>PMMA</td>
<td>Poly (methyl methacrylate)</td>
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<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
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<tr>
<td>RASFF</td>
<td>Rapid Alert System for Food and Feed</td>
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<tr>
<td>RCA</td>
<td>Rolling circle amplification</td>
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<td>Ribonucleic acid</td>
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<td>SDA</td>
<td>Strand displacement amplification</td>
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<td>Tris-EDTA</td>
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<td>TSG</td>
<td>Traditional Speciality Guaranteed</td>
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<td>W1</td>
<td>Washing buffer 1</td>
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<tr>
<td>μSPE</td>
<td>Microscale solid phase extraction</td>
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<tr>
<td>μTAS</td>
<td>Micro Total Analysis Systems</td>
</tr>
<tr>
<td>3SR</td>
<td>Self-sustained sequence replication</td>
</tr>
<tr>
<td>$\varepsilon$</td>
<td>Molar absorption coefficient [M-1cm-1]</td>
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CHAPTER 1. INTRODUCTION

According to the European Union (EU) definition, “quality” is the totality of characteristics of an entity that bears on its ability to satisfy stated and implied needs. On the other hand, “food safety” means the assurance that food will not cause adverse health effects to the final consumer.

In 1990s there were several safety crises in the food sector, such as the bovine spongiform encephalopathy (BSE), also known as “mad cow” disease, and some cases of dioxin contaminated feed.

The “mad cow” disease is a transmissible neurologic malady that occurred in cattle and some of its symptoms are altered behavior, uncoordinated pace and excessive reaction to touch or noise. It started in United Kingdom in 1986 and generated an epidemic event which had its peak in January 1993 (Nathanson, et al., 1997). The appearance of a new variant of Creutzfeldt-Jakob disease (CJD), which is a fatal degenerative neurologic disorder in humans, raised the question whether or not it could be related to BSE. This new variant of CJD appeared in 1994 in United Kingdom, which was the most affected country with BSE in the same period. The link between BSE and the variant of CJD in humans is now established, after different laboratory studies had been published exploring the origin of this new disease (Scott, et al., 1999).

The cases of dioxin contaminated feed affected several farms in Belgium. When the source of the problem was discovered more than 2500 farms could have been supplied with contaminated feed. It was a mixture of polychlorinated biphenyls (PCBs) and dioxins accidently added to a stock of fat used in animal feed production. The highest levels of contamination were found in poultry, especially in eggs, chicks and hens (Bernard, et al., 2002). These compounds are feed and food contaminants with high toxicity, having adverse effects in animals and humans health, which depending on exposure levels may include cancer.

This series of food safety crises highlighted the need for a change in the EU rules towards a more stringent food safety and quality control. In 1999, the European Commission (EC) published the White Paper on Food Safety (COM 719, 1999), which aimed to turn legislation into a more transparent set of rules in order to reinforce controls in the different stages of “farm to table” process and to guarantee a high level of human health and consumer protection. This “farm to table” or “farm to fork” approach was developed to assure quality and safety of food products from agricultural production to consumers market, introducing the concept of traceability.

1.1 Traceability

The EU integrated approach to food safety aims to assure a high level of food safety, animal health, animal welfare and plant health within the European Union through coherent farm-to-table measures and adequate monitoring, while ensuring the effective functioning of the internal market. One very important part of this
approach is traceability, which is the ability to track any food, feed, food-producing animal or substance that will be used for consumption, through all stages of production, processing and distribution (Health & Consumer Protection - Directorate General, 2007).

This ability is a preventive strategy regarding food quality and safety. If a good traceability system exists, it is easier to identify the origin of the problem when a food crisis occurs, putting in place all the corrective measures to stop it faster and taking preventive measures to avoid that it will happen in the future. The past food crises made evident the importance of being able to rapidly identify and isolate unsafe foodstuff, preventing them from continuing to reach the consumers market. Consequently, traceability through all the steps of food chain is critical to gain costumers confidence in the final product (Opara, 2003). The Figure 1.1 illustrates each step of the food supply chain (from farm to fork) where food safety and quality control practices must be applied.

With the world-wide integration of food supply chains, issues related with food safety and quality control are major concerns not only for the respective government authorities but also for the consumers themselves, which are now more aware for the consequences of a weak security and quality control in food industry.

Today, consumers want safe food but they do not want to be limited in choice or quality. They also wish to have more information about how their food is produced in order to be able to make informed choices. The results reported by a European Commission survey about European’s attitudes towards food safety and quality are illustrated in Figure 1.2. According to this survey, the majority of EU citizens believed that quality (65%), price (54%) and origin (34%) are very important when buying food, while their attitudes towards brands varied (Special Eurobarometer 389, 2012).

![Figure 1.1 – Steps of food supply chain (from farm to fork approach) (Will & Guenther, 2007).](image)

![Figure 1.2 – Results of the European Commission survey regarding the question about the importance that consumers give to some characteristic of food products when buying them (Special Eurobarometer 389, 2012).](image)
The perspective of consumers towards food has changed, however they have to rely on product labeling and advertising to get information about the nature of the product that they are buying. For this reason, it is extremely important that the product content and the label information provided are in conformity. This information is verified by processes of authentication.

1.2 Food Authenticity

Authentication, in the food quality context, is the process of validating or verifying the authenticity of a food product by confirming its identity. This process helps to prevent food fraud, which is the intentional false description of food composition carried out for financial gain. According to statistic studies this dishonest practice is on the rise. A report by the United Kingdom National Audit Office revealed that food fraud incidents have increased by two-thirds from 2009 to 2012 (National Audit Office, 2013).

Food authenticity issue, in particular in foods of added value (e.g. wine, olive oil, etc), has become one of the major concerns of the food industries and one important challenge for scientists, especially since it became a legitimate requirement in international food trade (European Commission, 2013). An example of a recent food fraud incident was the horsemeat scandal in 2013, which affected several countries in Europe. Horsemeat was detected in frozen beef-burgers and in beef-labeled ready meals. Tests showed that some of these products contained up to 100% horsemeat. The discovery of undeclared horsemeat in these foodstuff led to concerns about the presence of phenylbutazone, which is a painkiller used on horses that represents a potential risk for human health. Fortunately, this case was a matter of food fraud and not a matter of food safety (European Commission, 2013). However, other cases involved a health risk for consumers, such as the food fraud incident that happened in China in 2008. Milk powder was contaminated with melamine, which was used to boost protein levels, causing kidney failure in thousands of young children. The substitution of certain ingredients has also led to health risk in sensitized people due to the presence of undeclared allergenic ingredients. Unfortunately, food control authorities have to recall certain food products with more than desired frequency due to the presence of undeclared allergenic ingredients.

Although the majority of food fraud cases do not present risk to human health, this practice decreases consumer’s trust about the effectiveness of controls along the food chain, having a negative impact in agro-food sector and, consequently, in economy. In Table 1.1 are listed the top 10 products that are most at risk of food fraud, according to a report on the food crisis, fraud and controls (Committee on the Environment, Public Health and Food Safety, 2013).
The examples described before make evident the importance of food authenticity for consumers, food industry and authorities. Authentication contributes for preventing consumers of having health problems related with mislabeled allergenic ingredients as well as for nutritional quality problems for people with special diets. It also contributes for a fairer market, avoiding the replacement of expensive food ingredients for cheaper ones. Moreover, religion and taboo issues related with food in different societies and the consumption of endangered species can be prevented by authentication.

Lately, one of the big issues that have dominated public discussion in EU is the presence of genetically modified organisms (GMO) in food products. These organisms are plants, animals or microorganisms with genetically modified characteristics in order to enhance a specific property, for example to increase shelf life. However, there is little information about the possible long-term health effects of GMOs. For these reasons, it is mandatory to include in the labels whether or not a product contains GMOs in its composition, but that just can be verified with good authenticity and traceability systems.

Another advantage of authentication is the fact that it can help to preserve traditions. For example, in EU there is a protected geographical status (PGS) framework, which includes three types of regimes for protecting the authentication of local traditional food: Protected Designation of Origin (PDO), Protected Geographical Indication (PGI) and Traditional Speciality Guaranteed (TSG). This certification aims to protect the reputation of the regional foods and eliminate the unfair competition as well as prevent deceiving of consumers by non-genuine products (Rodríguez-Lázaro, 2013). There is also an Organic Farming Logo destined to foodstuff produced using approved organic methods. The applications of these different logos are described in Figure 1.3.

<table>
<thead>
<tr>
<th>Top</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Olive Oil</td>
</tr>
<tr>
<td>2</td>
<td>Fish</td>
</tr>
<tr>
<td>3</td>
<td>Organic Foods</td>
</tr>
<tr>
<td>4</td>
<td>Milk</td>
</tr>
<tr>
<td>5</td>
<td>Grains</td>
</tr>
<tr>
<td>6</td>
<td>Honey and maple syrup</td>
</tr>
<tr>
<td>7</td>
<td>Coffee and Tea</td>
</tr>
<tr>
<td>8</td>
<td>Spices</td>
</tr>
<tr>
<td>9</td>
<td>Wine</td>
</tr>
<tr>
<td>10</td>
<td>Fruit Juices</td>
</tr>
</tbody>
</table>

Table 1.1 – List of Top 10 food products that are most at risk of food fraud (Committee on the Environment, Public Health and Food Safety, 2013)
Certification of the origin of a food product and the raw materials used in its production is a guarantee of food quality because it allows verification of authenticity and discourages adulteration with components of lower value.

### 1.2.1 The Example of Olive Oil

In the last years, the integration of vegetable oils in human diet has increased significantly in part due to several publications reporting the benefits of olive oil for health, especially in prevention of coronary heart diseases (Huang & Sumpio, 2008). For these reasons and because it is expected that the olive oil global market continues to grow, this sector has a big influence in global economy (Directorate-General for Agriculture and Rural Development, 2012). However, some cases of virgin olive oil adulteration, using much cheaper refined olive oil, have been reported. Virgin olive oil is a premium product since it is obtained from the olives using only mechanical procedures without altering the oil in any way. Vegetable oils such as almond, hazelnut, maize, sunflower and palm are also used as adulterants of olive oil due to their lower prices (Frankel, 2010). Some of these vegetable oils are also potentially allergenic foods, such as hazelnut.

Olive oil is at the top of the list of the food products most at risk of food fraud, which makes evident that food authenticity and traceability are especially important for this sector.

### 1.2.2 The Role of DNA-Based Analysis

To perform the authentication process a set of different methods are used today, such as mass spectrometry, spectroscopy and separation techniques. The mass spectrometry is a method for measuring the mass-to-charge ratio of ions, which allows the identification of the sample composition by generating a mass spectrum with the masses of the sample compounds. It is often combined with other techniques. Spectroscopy
methods are based on the interaction of matter with radiation. The most used for food authentication are nuclear magnetic resonance (NMR) and near-infrared spectroscopy. Separation techniques include chromatographic methods, which allow physical separation of the different chemical substances in a mixture based on the different speeds at which they travel through a stationary material. This technique is more complex, time-consuming and expensive when compared with spectroscopy (Luykx & Ruth, 2007).

In the last years, the interest for DNA-based analysis of biological samples has grown exponentially. Many studies have been published applying these techniques to a range of different areas, such as in the field of forensic science and for pathogen detection in food, environmental and clinical samples. The application of these techniques to food authentication and detection of allergenic ingredients and GMOs has been increasing as well (Prado, et al., 2015). This increasing interest is mostly due to the high stability and durability of DNA when compared with proteins for example, and also because it is present in every cell of plants and animals which are the major constituents of food (Prado, et al., 2007). Moreover, since DNA is highly species-specific, the same DNA is present in the majority of the cells of an organism, which allows obtaining similar information from different tissues of the same organism (Brereton, 2013).

When considering a food sample like olive oil, DNA-based analysis is also very advantageous for cultivar identification. The quality of olive oil depends on the cultivar and the climatic conditions in the growing area, which justifies the difference in prices. The traceability of this product is difficult because chemical composition and phenotype may be changed due to environmental effects. So, contrary to DNA-analysis, chemical analysis and determination of biomorphological traits may not be able to identify the cultivars (Busconi, et al., 2003). While DNA analysis of olive oil has especially been used to authentication and traceability studies, other vegetable oils have been studied in order to identify the presence of GMO and, for these studies DNA is also considered the preferred target for analysis (Costa, et al., 2012).

The main steps for DNA-based analysis are extraction, amplification and quantification/detection of DNA. The extraction step is the process of purification of DNA from a sample. This step usually includes a cell lysis process, followed by the isolation of DNA from the complex mixture resulting from the lysis. The amplification step consists in amplifying a single copy or a few copies of DNA across several orders of magnitude. The most used techniques are based on polymerase chain reaction (PCR), which allows the exponential amplification of a DNA fragment and its mechanism is based on DNA replication in vivo. It is a highly specific, reproducible and sensitive method. However, this technology is highly limited by the presence of inhibitors, which can affect the reaction by decreasing or completely inhibiting it. For this reason, the amplification step is extremely dependent on the success of the extraction procedure, which should be able to recover the nucleic acids and, at the same time, to remove the PCR inhibitory compounds. The last step is the quantification/detection of DNA, which determines the concentration of a specific DNA fragment present in the initial mixture.

Complex matrices such as oil samples are particularly challenging for DNA extraction and posterior analysis. In particular, oil samples, besides the problem of being a lipidic matrix containing minor amounts of DNA, show low integrity of DNA as a consequence of the refining treatment needed in most vegetable oils
(Costa, et al., 2012), making DNA pre-concentration an interesting possibility during the extraction and purification procedure in order to facilitate DNA analysis.

1.2.3 Micro-scaled Technologies

Micro and nanotechnologies have been contributing to revolutionize many industry sectors, such as information technology, energy, environmental science and medicine. The food sector is not an exception and there is a wide range of applications for these miniaturized technologies and, in particular, for microfluidics.

Microfluidic systems are devices that can be used for sampling, monitoring, control and transport as well as for mixing, reaction, incubation, and analysis of small volumes of fluids. These systems are very popular in the so-called lab-on-a-chip (LOC) technology (Abgrall & Gué, 2007).

The main advantages of miniaturizing DNA analysis systems are the smaller volumes needed, which allows to consume less quantities of reagents as well as to improve the performance of the system by being faster and more sensitive. This type of technology also has the advantage of being more suitable for automation and decreasing the risk of contamination during the analysis process.

1.3 Motivation and Goals

The motivation for this work arises from the need of a rapid, simple, specific and sensitive system allowing reliable analysis results, through the different steps of the food chain. The systems for food safety and quality control from farm to fork applied today regarding DNA analysis are still expensive, relatively complicated and time-consuming. These techniques often require large specialized equipment and trained personnel as well as large sample sizes, limiting its application to centralized laboratory facilities of food industries.

This work intends to combine emerging technologies at microscale with the well-established molecular biology procedures, in order to improve DNA analysis applied to food safety and quality. With the growing application of the concept of food control from farm to fork, combining this two areas may be the solution to perform food safety and quality control measures in a simpler, faster and less expensive way and, thus, provide efficient analysis tools to the food industry and food control authorities.

With this in mind, the work produced for this thesis is focused in the development of an optimized DNA extraction method from food samples using a microscale solid phase extraction (μSPE) technique. This work is part of a larger research effort to develop a portable and integrated DNA analysis device for in situ applications in food industry sector, where is intended to include all the steps of DNA analysis in a single device.
As mentioned before, this work centers on the first step of DNA analysis: the DNA extraction. In this context, the main goal for this project is the optimization of each step of DNA extraction procedure in a microfluidic device with an embedded silica membrane designed in order to perform a solid phase extraction at microscale. The developed DNA extraction protocol included the optimization of the binding, washing and elution steps. This process has major importance for the rest of the DNA analysis stages, especially amplification with PCR due to its vulnerability to the presence of inhibitors. Since food samples represent a complex matrix containing not only DNA but also a mixture of many other compounds that can inhibit amplification, and considering that DNA present in food is often fragmented or degraded due to the many processing phases that some foods are put through, the adequate isolation of this nucleic acid can be really challenging. These challenges must be overcome in order to contribute for the development of a portable and integrated system that will change the food safety and quality control sector that we know today.
CHAPTER 2. LITERATURE REVIEW

2.1 DNA as a Biomarker

2.1.1 Structure of DNA

The deoxyribonucleic acid (DNA) contains the biological instructions that make each species unique. The Figure 2.1 describes the three-dimensional structure of DNA, which was defined by Watson and Crick in 1950s (Watson & Crick, 1953). This molecule consists of two single-strands hybridized together and coiled around a common axis, forming a double helix. Each single-strand is composed of monomers called nucleotides, which are made of three parts: a phosphate group, a sugar group (deoxyribose) and a nitrogenous base.

The nucleotides are linked together by covalent phospho-diester bonds that join the 5’ carbon of one deoxyribose group to the 3’ carbon of the next, forming the sugar-phosphate backbone. On a DNA molecule, four types of nitrogenous bases can be found: Adenine (A), Guanine (G), Cytosine (C) and Thymine (T). The first two are called purines, which are structures composed of two rings, while the last two are called pyrimidines, being composed of one single ring. Hybridization between the two DNA strands results from hydrogen bonding between complementary bases on opposing strands. A is complementary to T while G is complementary to C, which means that A always pairs with T, through two hydrogen bonds, and G always pairs with C, through three hydrogen bonds. If complementary strands are separated, they will spontaneously rebind in the right salt and temperature conditions. Due to this complementarity, the order of nucleotides on one strand predicts the order of nucleotides on the other one.

Figure 2.1 - DNA structure: (a) Double-stranded DNA helix (b) Nucleotide composition and base pair structure (Belk & Maier, 2007) Adapted.
2.1.2 Applicability in Food Safety and Quality Control Area

In the context of food safety and quality control, the main applications of DNA analysis methods include the identification of genetically modified organisms (GMOs), foodborne pathogen detection, food authenticity and the detection of allergenic ingredients in food products.

GMOs are organisms whose genetic material has been altered in order to enhance a specific property or introduce a new one. In agriculture sector there are genetically modified (GM) crops with traits that do not occur naturally in wild-type crops. Some examples of these new characteristics of GM crops are the resistance to pests, herbicides and environmental conditions as well as improvement of their nutrient composition and reduction of spoilage. The major GM crops cultivated are maize, soybeans, cotton, canola/rapeseed, sugar beets, alfalfa, papaya and squash (ISAAA, 2014). Some countries adopted regulations for GMO’s labeling however these regulations are different from country to country. In European Union, food products containing more than 0.9% of GMOs require respective labeling (Regulation (EC) No 1830/2003, Last updated:2011). This strict regulation makes DNA analysis of GMOs extremely important for these countries since the major difference between wild and GM crops is their genetic material.

Foodborne pathogen detection is a priority for guaranteeing food safety because a failure in this process may lead to dreadful consequences. According to the annual report of Rapid Alert System for Food and Feed (RASFF), concerning the total alerts in food and feed in 2014, around 33% of the alerts were about pathogenic microorganisms (RASFF, 2014). The conventional methods used for pathogen detection usually require long culture times and sometimes the microorganisms can be uncultivable, like some viruses. DNA-based methods can play a major role in the detection of foodborne pathogens since they are faster and can provide more information when compared with culture-based methods (Rasooly & Herold, 2008).

As mentioned before, food authenticity helps to prevent food fraud, which is a practice that has been rising in the last years and has very important economic and social consequences. Consumers have the right to know the correct information about the content of the food they buy, to be able to make informed choices for their diet, health, personal preferences, ethical and religious restrictions or cost. The presence of components of animal origin in vegetable matrices or the substitution of one or more ingredients with other ingredients of lower commercial value, are just some examples of common food frauds. Since foodstuff is mostly composed by ingredients of plant or animal origin, DNA-based methods can be used to identify these biological species and verify the authenticity of food products (Pascoal, et al., 2004) (Mafra, et al., 2008) (Madesis, et al., 2014).

Last but not least the detection of allergenic ingredients in foodstuff is another application for DNA-based methods. Food allergy is an abnormal immune response to food proteins and is characterized by the production of allergen-specific antibodies, called immunoglobulin E (IgE). There is no treatment for food allergies and the consequences of food mislabeling involving allergenic ingredients can be very serious. Thus, consumers with this problem need to know the content of food in order to avoid these substances (Rodríguez-Lázaro, 2013). These components must be listed when added intentionally but also when there is the possibility
of cross-contact during the production process. Food allergens are ideally detected using direct analytical methods, such as enzyme-linked immunosorbent assay (ELISA) which uses antibodies with specificity for the allergenic protein. Although the detection of the allergen itself would be desirable, this detection is not always feasible because, for example, the allergen’s chemical properties are not well characterized or the sensitivity of the available methodology is not adequate. In such cases, a different approach might be taken, such as choosing a marker indicative of the presence of the offending food (Poms, et al., 2004). For this purpose, DNA or proteins are usually targeted (van Hengel, 2007). The detection of specific DNA sequences from the allergenic ingredient can be more suitable when applied to complex food matrices or highly processed foods because in these cases proteins may be modified and, consequently, not detectable by protein analysis based methods (Prado, et al., 2015).

### 2.1.3 Complexity of Food Products

Food products are composed by several ingredients with different properties and the nature of each ingredient affects the efficiency of DNA extraction and, consequently, of subsequent DNA analysis. Having a mixture of components with different DNA contributions and different levels of DNA degradation, mixed in unknown ratios, makes a precise quantitative analysis nearly impossible (Weighardt, 2007). Moreover, some matrices have other constituents that may inhibit downstream analysis by PCR-based techniques. Since laboratories rarely receive complete compositional data on the products, the choice of the extraction technique is usually based on previous experience with similar samples. The extraction methods developed should be validated for different matrices and the range of matrices for which the respective protocol can be applied should be defined (Cankar, et al., 2006). Even samples from similar matrices, like grains of the same species, have differences regarding characteristics such as moisture, fiber, starch, and residues of chemicals (Lipp, et al., 2005). The majority of food products are processed and consequently they are even more complex because the same matrix varies from producer to producer depending on the processing procedure they use.

### 2.1.4 Susceptibility of DNA to processing

The processing procedure results in fragmentation and degradation of DNA and therefore limits the amount of DNA that can be extracted and the amount of amplifiable DNA. High temperatures and low pH are the factors which most break down the DNA. The effect of these and some other processes on DNA quality are described below.

- **Temperature**: High temperature processing results in the degradation of DNA which can be based on depurination (cleavage of purines resulting in release of the nitrogenous base – A or G) or deamination (removal of amine group). Temperatures above 100°C may cause considerable strand scission and irreversible loss of secondary structure. Some of the heat processes that degrade DNA are cooking, baking, drying and roasting. The autoclaving of food products at 121°C has a stronger effect on DNA degradation when compared with the cooking process what can be explained by the higher pressure,
increasing the destructive effect (Gryson, 2010). However in many cases the amplification remains possible (Prado, et al., 2002).

- **pH:** Acidic pH leads to cell lysis and consequently to the liberation of endogenous nucleases which degrade DNA. However this effect seems to be limited by the fact that the stability of these nucleases is lost before the stability of DNA, avoiding further breakdown. Low pH denatures DNA resulting into single-stranded DNA (ssDNA), which is still amplifiable. Just under strongly acidic conditions depurination can occur resulting in unsuccessful PCR. On the other hand, DNA is relatively stable at alkaline pH (Gryson, 2010).

- **Fermentation:** Long fermentation periods have a strong degradation effect on DNA according to the results obtained by Pan and Shih. This study consisted on the detection of transgenic DNA in Miso during a 6-month period of fermentation. The results were positive until the 100th day of fermentation and it was observed a gradual decreasing of DNA detected. After the 120th day the results were unreliable and false-negatives were obtained (Pan & Shih, 2003). This strong degradation might be explained by the changes in temperature and pH during the process of fermentation as well as by the presence of nucleases derived from microorganisms.

Processes such as refining of oils and enzymatic treatments also have a big effect on DNA degradation. On the other hand, DNA is highly resistant to mechanical treatments (Gryson, 2010). In conclusion, many processing procedures affect the quality of the DNA, but in many cases PCR amplification remains possible.

Factors related with the design of the PCR method used have been pointed out as well as important keys for the success of the analytical procedure, among these factors amplicon size has been indicated as one of the critical parameters that affect amplification efficiency (Prado, et al., 2007) (Hird, et al., 2006).

### 2.1.5 Advantages and Limitations

The analytical techniques mentioned in the previous chapter, such as mass spectrometry, nuclear magnetic resonance (NMR), near-infrared spectroscopy or chromatography, have been successfully used for identification of food frauds. However, the information achieved using these techniques is more suited for geographical origin authentication purposes, lacking in its potential for species-specific identification (Luykx & Ruth, 2007). Thus, a combination of different analytical techniques could be more useful than relying on one single method.

Analytical methods to identify species usually rely on techniques for protein detection or on DNA-based methods. The protein detection can be achieved using different analytical approaches, including electrophoresis and immunological methods, among others. These techniques are very sensitive, accurate and easy to apply but they are only applicable to raw and unprocessed foods. This limitation exists because, as mentioned before, many target proteins are degraded by processing procedures. So, if the proteins do not have the same characteristics as the one for which the antibodies were developed, those antibodies will not be able to recognize the specific protein.
Some advantages for the use of DNA-based methods in food analysis are described below.

- **Higher stability and durability than proteins or ribonucleic acid (RNA).** Denaturation of DNA at high temperatures results in the separation of the double strand into two single strands, which occurs when the hydrogen bonds between the strands are broken, but DNA strands can realign when the conditions are restored. In contrast, proteins denature at lower temperatures than DNA and most of them lose their function irreversibly. In the case of RNA, its sugar group is a ribose (instead of deoxyribose like in DNA) and this sugar has a hydroxyl group (-OH) in 2’ the position, which makes RNA more prone to hydrolysis than DNA (Khanna, 2009).

- **High specificity and sensitivity.** The concept of specificity is related with how much the “model” rejects the “objects” of different categories (based on DNA sequence of interest), while sensitivity is related with how much the “model” accepts the “objects” of the same category (amplification of a specific DNA sequence) (Sun, 2008). The differences in the DNA sequence between species make it a key tool for species authentication, allowing the development of very specific analytical methods. When compared with proteins, DNA can provide more information through the acquisition of sequence data for the verification of food species, due to the degeneracy of the genetic code and the presence of many non-coding regions (Brereton, 2013). At the same time, another important advantage of the use of DNA is the possibility of amplifying the number of copies of the initial DNA target by PCR or other amplification techniques, which enables higher sensitivity, as well as the feasibility of quantification by the use of quantitative PCR (qPCR).

- **Not affected by biological variations (seasonal and geographical impacts).** Genetic material is present in every cell of plants or animals which are the major constituents of food, thus similar information can be obtained from different tissues of the same organism, in contrast with proteins whose expression might be affected by certain external factors.

- **Standardization of DNA-based methods** is simpler due to their intrinsic characteristics, comparing with immunological methods. Antibodies from different suppliers might result in different assay performances, unlikely for DNA (Brereton, 2013).

There are also some limitations related with the use of DNA-based methods in food analysis. Each food matrix presents different problems, however the major limitations to the application of DNA-based methods are the loss of DNA integrity due to processing procedures during the production of food (Hird, et al., 2006), the risk of cross-over contamination along the several steps of DNA analysis and the presence of compounds which prevents DNA detection (Nolan, et al., 2006), among which the inhibitors of PCR that are discussed further in this chapter.
2.2 DNA Extraction

The purpose of DNA extraction is to obtain DNA in a relatively purified form in order to be used for further investigation. In this subchapter some of the most used techniques for DNA extraction will be described, at both traditional bench scale and micro scale.

2.2.1 Sample Preparation and Cell Lysis

Before starting the extraction of DNA, the homogeneity and representativeness of the sample must be assured. If the sample is homogeneous such as a single ingredient food product (e.g. sesame seeds) the whole sample may be considered representative. However, for heterogeneous samples (e.g. pizza) the representativeness is a critical step. Once a representative sample is selected, it must be homogenized and a technique for cell lysis must be applied.

There are many ways to achieve lysis in order to release the DNA within the cell, which include mechanical, enzymatic and chemical methods. Mechanical methods are more effective and give a more uniform cell disruption. Some examples are thermal shock, bead-mill homogenization, bead beating and sonication, but the simplest method is grinding in liquid nitrogen using a mortar and pestle (Chauhan & Varma, 2009). Chemical and enzymatic methods are relatively gentle, producing limited shearing of DNA. Chemical methods usually include detergents, which dissolve the membrane proteins and lipids (e.g. sodium dodecyl sulfate (SDS), Triton) and chaotropic agents (e.g. guanidine thiocyanate). Chaotropic agents can disrupt the structure of water, making it a less hydrophilic environment and weakening the hydrophobic interactions, this way they are able to bring some hydrophobic compounds into aqueous solutions (Ghosal & Srivastava, 2009). Enzymatic methods use enzymes to lyse cells. Some examples are lysozyme, which is used to disrupt bacterial cell walls, and proteases (e.g. proteinase K), which are used to digest proteins. These three types of cell disruption methods are usually combined in order to achieve a more effective lysis.

2.2.2 Traditional Solid-Phase DNA Extraction

Prior to the development of solid-phase methods, the Cesium Chloride (CsCl) gradient and the phenol-chloroform extraction techniques were used. The CsCl gradient method consists in the formation of a density gradient, through which the nucleic acids migrate until reaching neutral buoyancy, the isopycnic point. This way the DNA is separated from the other components. An intercalating dye (usually ethidium bromide) is used to allow visualization of the DNA band after centrifugation (Weising, et al., 1995). The phenol-chloroform extraction involves mixing of a phenol-chloroform solution with an aqueous sample, resulting in the denaturation of proteins and other contaminants to the lower organic phase, while DNA remains in the upper aqueous phase (Price, et al., 2009). However these techniques use some undesirable reagents, such as ethidium bromide, which is a mutagen, and phenol due to its toxicity.
Solid-phase extraction (SPE) methods are easier to perform than liquid/liquid extraction, less time-consuming and can be automated. SPE is a sample preparation procedure, quite used in analytical chemistry, by which compounds that are dissolved or suspended in a liquid mixture are separated from other compounds in the mixture according to their physical and chemical properties. Compounds of interest are retained on a stationary phase due to their higher affinity (Poole, 2003). Analytical laboratories frequently use solid phase extraction to concentrate and purify samples for analysis.

In Figure 2.2 are described the main steps of a DNA extraction procedure based on solid-phase extraction, which include lysis, binding, washing and elution. The lysis step is used to break down the cell wall and cellular membranes of the sample and it usually includes mechanical forces (e.g. grinding) and the addition of detergents. The binding step consists in binding the DNA to the solid phase selected for the extraction, under the right salt concentration and pH conditions. After the binding, proteins and other unbound impurities are removed during the washing step. Finally, the DNA is eluted under adequate salt concentration and pH conditions in a relative pure form.

Different SPE matrices can be used for DNA extraction, such as glass fiber, silicon dioxide and diatomaceous earth, but the most commonly used is silica.

**Principle of Silica Matrices**

The main driving forces for DNA adsorption to silica are the electrostatic forces, dehydration, and hydrogen bond formation. DNA has a large negative surface charge density and silica is also negatively charged at basic and near neutral pH (Melzak, et al., 1996). Consequently, in aqueous solutions there is an electrostatic repulsion between them, inhibiting the generation of hydrogen bonds. This repulsion can be overcome by high ionic strength conditions, which shields the negative charges at both DNA and silica surfaces. The decrease of pH value promotes the protonation of phosphate groups of DNA and silanol groups of silica, which intensively increases the binding sites for DNA interaction with silica (Li, et al., 2012). As a result, silica and DNA dehydration effects and intermolecular hydrogen bond formation become able to overcome this weakened electrostatic repulsion and drive DNA adsorption to the silica surface.
Melzak et al. described this adsorption reaction by equation (2.1) (Melzak, et al., 1996).

\[
\text{DNA (hydrated) + silica (hydrated) + counterions} \leftrightarrow \text{neutral DNA/silica complex + water} \quad (2.1)
\]

The amount of free water molecules decreases due to the formation of hydrated ions from the salt, driving the reaction to the right. Although non-chaotropic salts can be used, this effect is enhanced by the use of chaotropic salts since they can disrupt the hydrogen bonding network between water molecules, weakening the hydrophobic effect (Salvi, et al., 2005). Consequently, the consistent formation of hydration shell around the macromolecules is eliminated. The classification of ions as chaotropic is given by the Hofmeister series illustrated in Figure 2.3.

![Hofmeister series](image)

An alternative model to describe the bond between the DNA and the silica membrane is the formation of a cation bridge between the negatively charged DNA backbone and the negatively charged silica surface (Cultek, s.d.). Figure 2.4 illustrates the hydration shell formed in absence of chaotropic salts (A), as well as the hydrogen bond formed in their presence (B) and the alternative model with the cation bridge formation (C).

![Models](image)
Silica based DNA extraction kits are widespread, being produced by Clontech (Nucleo-Spin™), Mo Bio Laboratories (UltraClean™ BloodSpin™), Qiagen (QiaAmp™), Promega (Wizard™), Epoch Biolabs (EconoSpin™), Sigma Aldrich (GenElute™) and others (Price, et al., 2009).

2.2.3 Other Traditional DNA Extraction Methods

Besides the resin binding methods, which include the silica-based solid phase extraction described before, there are other techniques available for DNA extraction at bench scale. The other most common techniques are precipitation methods and magnetic particles (Sforza, 2013).

One of the most recurrent precipitation methods described in literature is the precipitation of DNA with cetyltrimethyl ammonium bromide (CTAB), which is a surfactant. This method is based on the selective precipitation of nucleic acids using CTAB in a solution of low ionic strength, resulting in the separation of the precipitated DNA from contaminants (e.g. polysaccharides) that remain soluble. The precipitate is then dissolved in a higher ionic strength solution and the non-polar compounds are removed by extraction to chloroform (Rodríguez-Lázaro, 2013). Although the DNA obtained is not of high purity, it is generally of sufficient quality for amplification by PCR and it is a simple and easy to control method (Sforza, 2013).

Magnetic particle kits have been developed and used for DNA extraction procedures, bringing some advantages compared with non-magnetic systems. Some of these advantages are the ability to suspend the particles freely in the sample solution, maximizing the interactions, and the ability to collect the particles using a magnetic field instead of a centrifugation or filtration process (Reinholt & Baeumner, 2014). Commercially available magnetic particles can be obtained from a variety of companies (e.g. Wizard Magnetic™ from Promega) and they can be coated with functional groups or be left uncoated. The matrices are generally based on silica, porous glass, cellulose, agarose, polystyrene and silane (Berensmeier, 2006).

2.2.4 Microscale Solid-Phase DNA Extraction

Methods for DNA analysis at bench scale are well-established, however they require large sample volumes, large specialized equipment, trained personnel and are time-consuming and expensive. These characteristics limit their portability and, consequently, centralized laboratory facilities are required (Reinholt & Baeumner, 2014). The miniaturization of these methods has been developed in order to overcome this big limitation.

DNA extraction is a critical step and should obtain sufficiently concentrated and high quality DNA for downstream processes, which may use very small volumes. Silica-based DNA extraction is one of the most suitable methods for adaptation to microchips because they do not necessarily require hands-on-processing steps (e.g. centrifugation) (Price, et al., 2009). The principle of this method at micro scale is the same as at
bench scale, starting with a non-specific binding of nucleic acids to silica under high salt concentration, followed by a washing step using an organic solvent (e.g., ethanol, 2-propanol) to remove contaminants. The nucleic acids bound to silica are then eluted using a low ionic strength solution.

There are different types of microfluidic designs for silica-based DNA extraction, which include packed silica beads, matrices containing silica beads, silica microstructures, silica coated paramagnetic beads and silica surfaces (Reinholt & Baeumner, 2014).

Systems using packed silica beads incorporate the beads into microcapillaries, confining them within the channel while allowing fluid flow. This is accomplished by designing a larger inlet compared to the outlet, which is smaller than the beads size, forming a weir structure. Wolfe et al. tested this design and DNA extraction was successfully achieved, being suitable for PCR application (Wolfe, et al., 2002). Although the packed silica beads design has a simple concept and is well-established at bench scale, there are some drawbacks associated. Some of these drawbacks are the bead compaction, which decreases the flow because beads tend to pack more tightly, the poor reproducibility of results from run to run and the variability from chip to chip as a result of silica bead packing in the channel (Price, et al., 2009).

Matrices containing silica beads were developed as an improvement on systems using packed silica beads and it is based on the confinement of silica beads within porous materials, such as sol-gel matrices. Sols are liquid colloidal suspensions which, when catalyzed, turn into gel, forming solid structures. Since the time for condensation can be controlled, the sol-gel precursor can be flowed into the microchannel as a liquid and then gelled in place to form the necessary solid matrix, incorporating the beads (Wolfe, et al., 2002). Wolfe et al. tested this approach, obtaining amplifiable DNA with better efficiency, improved reproducibility and stability compared with packed silica beads (Wolfe, et al., 2002). The main advantages of this approach are the large surface area to volume ratio and the systems being generally reusable. However, it requires post-fabrication processing and high fabrication temperatures, which needs expensive materials for the microfluidic platform (Reinholt & Baeumner, 2014).

Silica microstructures, which include micropillars or other complex structures, increase the surface area to volume ratio and mixing within the device. These characteristics are defined by the size, density and shape of the microstructures. The selection of these design characteristics should be made according to the sample size and concentration. DNA extraction efficiency increases with the surface area to volume ratio, but decreases with the flow rate. Different shapes of microstructures can be used to define the flow pattern (Reinholt & Baeumner, 2014). This technique has been used for DNA extraction and different shapes of micro structures have also been described in literature. Some advantages of this design are the possibility to have higher flow rates, reusability and the fact that no post-fabrication processing is needed. However these systems can be expensive due to the complex manufacturing process and materials required (Reinholt & Baeumner, 2014).

Silica-coated paramagnetic beads have also been developed and used for DNA extraction at micro scale. This approach has several advantages over silica packed beads because instead of being packed and confined in
a chamber, paramagnetic beads can be free in solution and then collected using a magnetic field. This main difference increases the surface area for DNA binding and there is no issue of clogging. However paramagnetic beads are expensive and the need for a magnetic field brings some limitations to the microchip design, namely regarding the type of material and its thickness (Reinholt & Baeumner, 2014).

**Silica membranes** are another format for silica-based DNA extraction at micro scale, consisting of a network of glass fibers. The membrane is incorporated in the microfluidic system within a chamber, being physically secure, and the fluid passes through the membrane during the extraction process. This is a very common approach due to the easy integration of the membrane into the microfluidic device as well as its similarities with the bench scale approach. *Kim et al.* tested a microfluidic system using silica membranes and the results demonstrated efficient DNA extraction, suitable for downstream amplification process (Kim, et al., 2012).

In Figure 2.5 are illustrated some of the microfluidic designs for silica-based DNA extraction described before.

![A: Microscopy image of silica beads within a microchannel (10x magnification)](image1)  
![B: Microscopy image of silica beads incorporated into a sol-gel matrix within a microchannel (10x magnification)](image2)  
![C: Silica micropillar array](image3)  
![D: Silica membranes of different sizes](image4)  
![E (a) – FE-SEM image of magnetic beads coated with silica](image5)  
![E (b) – FE-SEM image of magnetic beads coated with silica (10x magnification)](image6)

**Figure 2.5 – Types of designs for silica-based DNA extraction:** A – Microscopy image of silica beads within a microchannel (10x magnification); B - Microscopy image of silica beads incorporated into a sol-gel matrix within a microchannel (10x magnification); C – Silica micropillar array; D – Silica membranes of different sizes; E (a) – FE-SEM image of magnetic beads coated with silica; E (b) – FE-SEM image of magnetic beads coated with silica (10x magnification).

Besides silica, there are many other matrices that can be used for solid phase extraction. For example, the solid-phase can be coated with specific oligonucleotide sequences, allowing complementary hybridization of the target nucleic acids, or can have matrices with switchable charges coating the surface, which allows nucleic acids extraction based on pH and avoids the use of inhibitors of the downstream amplification process (Reinholt & Baeumner, 2014) (Price, et al., 2009). Chitosan is a polymer that is protonated at pH 5, allowing DNA binding, and neutralized at pH 9, allowing its elution. This polymer has been used for nucleic acids extraction based on these switchable charges and has demonstrated sample-to-sample and chip-to-chip reproducibility (Reedy, et al., 2011).
2.2.5 Other Microscale DNA Extraction Methods

There are other methods for DNA extraction that do not use a solid-phase, namely electrophoretic techniques and organic solvents.

Electrophoretic techniques use mobility in an electric field to separate contaminants from the nucleic acids. These methods have several advantages over solid-phase extraction, namely the fact that any substrate material can be used, solutions that may inhibit downstream amplification process are avoided and a voltage source is used instead of external pumps, which decreases the device size. However, the electrophoretic mobility of the nucleic acids to be isolated must be known (Reinholt & Baeumner, 2014).

The isolation of nucleic acids using organic solvents is well-established at bench scale, namely the phenol-chloroform extraction. This method has been integrated in microdevices, allowing successful extraction of nucleic acids and removal of contaminants (Zhang, et al., 2013). This type of isolation has higher purification efficiency than solid-phase methods however the use of hazardous organic solvents is required, demanding safe handling and disposal (Reinholt & Baeumner, 2014).

2.3 Amplifiability of the Isolated DNA

The amplifiability of DNA is determined by its purity, quantity and quality. The purity of DNA is related with the presence of contaminants, which can be originally present in the material under examination, such as lipids, polysaccharides and proteins, or can result from chemicals used during the DNA extraction method selected, such as phenol, ethanol or isopropanol. The quantity of DNA extracted from a sample has an impact on the detection and quantification limits of the amplification process. DNA quality depends on its degree of fragmentation, which varies according to the material under examination, degree of processing and the DNA extraction method applied.

In this subchapter some of the techniques used to determine the purity, quantity and quality of the extracted DNA will be discussed.

2.3.1 DNA Purity and Quantification

UV spectrophotometry and fluorescent spectrometry are two techniques that can be used for DNA quantification. The first one can also be used to determine the purity of extracted DNA.
2.3.1.1. UV-Vis Spectrophotometry

An Ultraviolet-Visible (UV-Vis) spectrophotometer is employed to measure the amount of light that a sample absorbs from the near ultraviolet to the near infrared spectral region (190nm – 840nm). Using the Lambert-Beer Law, described by equation (2.2), it is possible to relate the amount of light absorbed to the concentration of the absorbing molecule.

\[ A = \varepsilon b C \tag{2.2} \]

Where \( A \) is the absorbance, \( \varepsilon \) corresponds to the molar absorption coefficient \( (\text{M}^{-1}\text{cm}^{-1}) \), \( b \) is the path length of the sample and \( C \) represents the concentration of the compound in solution \( (\text{M}) \).

Nucleic acids in solution absorb UV light in the range from 210 nm to 300nm with an absorption maximum at 260nm. Since nucleic acids do not absorb at 320nm, reading at this wavelength is informative for the determination of background absorption due to light scattering and UV-active compounds (Rodríguez-Lázaro, 2013). RNA also has its absorption maximum at 260nm so it must be removed enzymatically during the DNA extraction process because the presence of compounds contributing to the total measurement at 260nm may result in the overestimation of DNA quantity.

To evaluate DNA purity, the determination of absorbance at 280nm is often carried out because this is the absorption maximum of proteins. The ratio of absorbances at 260nm and 280nm (A260/A280) has been used as a measure of purity of DNA. As a result, a DNA sample can be considered “pure” if it has an A260/A280 ratio between 1.7 and 2.0. Lower ratios indicate that more contaminants are present, but do not mean that the DNA is not suitable for downstream applications. Absorbance at 230nm is also measured in order to indicate if other contaminants are present in solution, such as organic compounds or chaotropic salts. The A260/A230 ratio for “pure” nucleic acid is often higher than the respective A260/A280 ratio, being commonly in the range of 2.0-2.2 (Thermo Fisher Scientific, s.d.).

This technique is very simple but has some disadvantages, namely its low sensitivity, the need for removal of RNA for precise DNA quantification as well as the contribution of signal from single-stranded DNA (ssDNA).

2.3.1.2. Fluorescent Spectrometry

Fluorescent spectrometry is based on the excitation of specific compounds from a sample using a beam of light, causing them to reemit light afterwards, usually at a longer wavelength. The concentration of unknown samples is then calculated based on comparison to a standard curve obtained from samples of known concentration.

This method is commonly used for DNA quantification, being more sensitive than absorbance methods due to the use of DNA-binding dyes, such as PicoGreen, SYBRGreen and Hoechst dyes. For instance, PicoGreen is an
intercalating dye which exhibits an emission maximum at 530nm when bound selectively to double-stranded DNA (dsDNA) (Thermo Fisher Scientific, s.d.). This way, the contribution of signal from ssDNA and other contaminants is reduced, overcoming some of the disadvantages of absorbance method. However, even precise DNA quantification may not provide enough information about the amplifiability of DNA because it also depends on the degree of fragmentation or the presence of other inhibitors of amplification (Rodríguez-Lázaro, 2013).

2.3.2. DNA Quality

2.3.2.1. Agarose Gel Electrophoresis

Gel electrophoresis uses an electrical current in order to separate biological molecules based on size. A sample of the extracted DNA is loaded into a well of the agarose gel and then exposed to an electric field. Then, the negatively charged DNA migrates toward the positive electrode and, since small DNA fragments migrate faster, the DNA is separated by size. In order to visualize the DNA in the gel is necessary to use a fluorescent dye. Ethidium bromide is commonly used for that purpose but, due to its toxicity, has been replaced by less hazardous reagents, namely GelRed™, GelGreen™, MidoriGreen™ and others. To determine the size of DNA fragments in an electrophoresis gel a set of DNA molecules of known length, called DNA ladder, is required. This technique can be used to analyse the quality of DNA, regarding its degree of fragmentation (Prado, et al., 2004).

The presence of an intense, high molecular weight band in the gel indicates DNA with minimal degradation while degraded DNA can be visualized as a smear towards the lower molecular weight region of the gel. In the context of food processing, DNA can be characterized as high (>20 kbp or unprocessed), medium (mainly 20–0.5 kbp), low (mainly 500–100 bp) or very low (<100 bp) molecular weight (Gryson, 2010). For successful downstream amplification the target region must be complete, therefore, if the food samples are highly processed, short fragments should be targeted (Prado, et al., 2007) (Costa, et al., 2012).

This method gives a very rapid and sensitive estimation of DNA degradation, however the result obtained with agarose gel electrophoresis does not necessarily correspond with the following amplification result because inhibitors of amplification can still be present even if the DNA quality is good (Gryson, 2010).

2.4. DNA Amplification

After the extraction, DNA must be amplified in order to increase exponentially the number of copies of a particular DNA sequence for subsequent detection. The most common method used for this purpose is the Polymerase Chain Reaction (PCR), which is based on the principle of DNA replication in vivo and relies on a
Several PCR variants have also been developed in order to optimize this technique. In addition there are isothermal techniques also available for DNA amplification.

In this subchapter some of these amplification methods will be discussed as well as its ability to be incorporated in a microfluidic setting.

2.4.1. Conventional PCR

The principle of PCR was first described by Kleppe et al. in 1971, and further developed by Kary Mullis, who was awarded together with Michael Smith the Nobel Prize in Chemistry in 1993 (Saunders, 2008). Since then, this method has been used in different areas, including food safety and quality control, due to its versatility, sensitivity and specificity.

The Principle of PCR

PCR is based on an exponential amplification of a DNA fragment through a temperature dependant enzymatic reaction. To perform this technique some components are required, namely the extracted DNA template containing the target DNA region, a thermal stable DNA Polymerase (usually Taq DNA polymerase) which is an enzyme responsible for the assembling of nucleotides, two primers which are short nucleic acid sequences that serve as starting point for the DNA synthesis, deoxynucleotides (dNTPs) which include the four different types of nucleotides (dATP, dTTP, dGTP and dCTP), a divalent cation such as magnesium, which is a cofactor of DNA polymerase, and a buffer solution to provide a suitable chemical environment (Loeffelholz & Deng, s.d.).

This method includes three steps, namely denaturation, annealing and extension. One round of these steps is referred to as a PCR cycle, which is illustrated in Figure 2.6. During the denaturation step, the temperature is increased to 93-96°C breaking the hydrogen bonds responsible for holding the strands of the extracted dsDNA together. After obtaining the single-stranded DNA, the temperature is reduced to 55-65°C for the annealing step, where the two primers anneal to the complementary regions, flanking the DNA sequence to be amplified. When primers come in contact with a perfectly complementary target sequence, the bond that forms is sufficiently stable to allow DNA polymerase to bind and initiate the third step of the cycle. The extension step is carried out by DNA polymerase which adds dNTPs from 5’ to 3’ reading the template DNA from 3’ to 5’ and resulting in the synthesis of a DNA strand complementary to the sequence of interest (Rodríguez-Lázaro, 2013). This step occurs at 72°C which is the optimum temperature for DNA polymerase activity.

After each cycle, the newly synthesized DNA strands serve as template in the next cycle. Thus, considering an efficiency of 100%, at the end of each cycle the amount of DNA target is doubled, leading to exponential amplification.
The analysis of the PCR product relies on its size or sequence. The size of the amplicon (amplified sequence) can be determined by gel electrophoresis, which is inexpensive and simple to implement. However, since molecules of approximately the same size cannot be distinguished by electrophoresis, this detection method might not be enough in some cases. The analysis of the amplicon based on its sequence is far more reliable and can be achieved using probe hybridization assays with fluorescent markers (Saunders, 2008). However, this detection method is time-consuming and lacks in available automation.

2.4.2. Variants of PCR

Several variants of PCR have been developed in order to optimize this amplification method, such as multiplex PCR and real-time PCR.

**Multiplex PCR** allows the simultaneous amplification of more than one target sequence in a single reaction by using more than one set of primers. This variant is advantageous because in a single test-run is possible to achieve more information about the extracted DNA, for instance, this technique can be used to identify different foodborne microorganisms or different food allergens in the sample with a single PCR test (Chen, et al., 2012) (Hubalkova & Rencova, 2011). However, this method requires careful attention to the set of primers.
selected because their annealing temperatures must be the same, their sequences must be sufficiently different in order to avoid dimerization (primers binding to each other) or competition for reagents on the mixture of amplification among primers sets, and the amplicons must have different sizes (Loeffelholz & Deng, s.d.).

**Real-time PCR or quantitative PCR (qPCR)** allows monitoring of the synthesis of the amplicons during the reaction in real-time by using fluorescence. This method is more advantageous compared with the conventional PCR because it greatly simplifies the amplicon detection, not only at the end of the reaction, as occurs in conventional PCR, but also during the process, resulting in the formation of an amplification curve that can be used to quantify the initial amount of the target DNA in the sample (Rodríguez-Lázaro, 2013). An amplification curve is illustrated in Figure 2.7, presenting the three phases of a PCR reaction: initiation, exponential and plateau phases. The initiation phase occurs during the first PCR cycles, when the emitted fluorescence cannot be distinguished from the baseline. In the second phase an exponential increase of the emitted fluorescence is observed and, in the plateau phase, which is the last phase of the amplification curve, no more PCR product accumulates due to the exhaustion of reagents and enzyme. The threshold of the real-time PCR reaction is the level of signal that reflects a statistically significant increase over the calculated baseline signal and the threshold cycle (C<sub>t</sub>) is the cycle number at which the fluorescent signal of the reaction crosses the threshold. The quantification of target DNA in the sample can then be determined by interpolation of the resulting C<sub>t</sub> value in a linear standard curve obtained from dilution series of known template concentrations.

![Figure 2.7 – Phases of a PCR amplification curve (Rodríguez-Lázaro, 2013).](image)

The main component that allows this real-time monitoring is the fluorescence, which can be unspecific or sequence specific. Unspecific fluorescent molecules, such as SYBRGreen®, emit fluorescence when bound to dsDNA, so an increase in the fluorescence signal is observed during the extension step and it decreases when denaturation step begins. Consequently, fluorescence measurements must be done at the end of the extension
step of each cycle. This type of detection does not need a probe synthesis, being less expensive, but its specificity is entirely determined by the primers. Sequence-specific probes, which are single-stranded DNA probes designed to hybridize to the internal region amplified by the primers, can also be used. There are different designs for the probes but essentially they can be classified into two main groups: hydrolysis probes and hybridization probes. The main difference between them is that the first ones are cleaved by DNA polymerase after hybridization while the second ones, instead of being hydrolyzed, are subjected to a change in their structure when bound to the DNA, allowing emission of fluorescence. As an example, one probe commonly used is the TaqMan®, which is a hydrolysis probe and is illustrated in Figure 2.8. One of the nucleotides of the probe is labeled with a fluorescent molecule, called reporter, and another one is labeled with a fluorescence quencher molecule, therefore, when these two molecules are close to each other, the quencher absorbs the fluorescence emitted by the reporter. After the hybridization of the probe, the extension step of PCR begins and the DNA polymerase starts to synthesize the complementary strand. When the DNA polymerase encounters the dsDNA formed by the probe hybridization, it disassembles the probe and replaces all the nucleotides, resulting in the separation of the reporter molecule from the quencher and, consequently, fluorescence can be emitted.

![Figure 2.8 – Principle of detection using TaqMan® probes (Rodríguez-Lázaro, 2013).](image)

The real-time PCR can be integrated with the multiplex PCR using different fluorescent molecules for each target sequence.

The major advantages of real-time PCR over the conventional method are the high reliability and sensitivity, the lower risk of contamination due to its closed-tube format, the possibility of using shorter fragments (Prado, et al., 2007) and the ability to perform analysis in a faster and easier way. The type of detection used in real-time PCR makes this technique more suitable for automation and integration in a microfluidic setting.
2.4.3. PCR inhibitors

Some limitations must be considered regarding the PCR technique, namely the fact that any form of contamination of the sample can produce misleading results, prior sequence data is required in order to design the primers and incorrect nucleotides can be incorporated in the complementary chain by DNA polymerase, resulting in mutations in the DNA fragment. In addition, primers can bind non-specifically to sequences that are similar to the target (Garibyan & Avashia, 2013). Another limitation of this method is the interference of PCR inhibitors, which include a large variety of compounds that can reduce the amplification efficiency or inhibit it completely (Nolan, et al., 2006). These compounds can be originally present in the sample or can be added during the DNA extraction process. Food samples are complex matrices and several PCR inhibitors can be found in them, such as polysaccharides, proteases and calcium ions. Moreover the majority of the DNA extraction methods use chemical reagents that may inhibit PCR, such as chaotropic salts, detergents, ethylenediaminetetraacetic acid (EDTA), phenol, isopropanol, ethanol and excess salts like potassium chloride (KCl) and sodium chloride (NaCl), among others (Demeke & Jenkins, 2010). Therefore, it is critical to develop a method that can efficiently remove these inhibitory substances that interfere with the amplification of the target DNA.

PCR inhibitors can affect directly the target DNA by leading to its precipitation or denaturation and can also interfere with the DNA polymerase by blocking its activity. DNA polymerases have cofactor requirements, for example, magnesium ($\text{Mg}^{2+}$) is a critical cofactor for this enzyme, so compounds that reduce its availability or interfere with the binding of this cofactor to the DNA polymerase can also inhibit PCR (Demeke & Jenkins, 2010).

An inhibition test can be performed by adding a known amount of non-endogenous DNA to the sample for amplification as an internal positive control. This control can be used in real-time PCR, providing quantitative information about its performance. In order to overcome inhibition, the extracted DNA can be diluted, reducing the inhibitors concentration however the DNA concentration also decreases, which may reduce the sensitivity of the reaction. Another way to overcome PCR inhibition is by using a DNA polymerase less susceptible to the effects of the inhibitors or by adding facilitators to improve the activity of the DNA polymerase, such as bovine serum albumin (Klančnik, et al., 2012).

2.4.4. Isothermal Amplification Techniques

Several isothermal amplification techniques have been developed over the last years, namely self-sustained sequence replication (3SR), nucleic acid sequence based amplification (NASBA), strand displacement amplification (SDA) and rolling circle amplification (RCA). Each one of these techniques has its own mechanism to re-initiate new rounds of DNA synthesis and can amplify nucleic acids to a magnitude similar to PCR. However, they require precision instruments and/or complex methods for product detection (Fakruddin, 2011).
More recently, a new isothermal amplification technique, called loop-mediated isothermal amplification (LAMP), was developed, promising to be a method of choice for DNA amplification (Notomi, et al., 2000).

**Principle of LAMP**

The chemical pathway for nucleic acid amplification in LAMP is complex, employing 4 primers which recognize 6 specific sequences as described in Table 2.1.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Description</th>
<th>Illustration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FIP</strong> (Forward Inner Primer)</td>
<td>Composed by the F1c region at the 5' end and the F2 region at the 3' end, which is complementary to the F2c region of DNA</td>
<td>![FIP Illustration]</td>
</tr>
<tr>
<td><strong>F3 Primer</strong> (Forward Outer Primer)</td>
<td>Composed by the F3 region that is complementary to the F3c region of DNA</td>
<td>![F3 Primer Illustration]</td>
</tr>
<tr>
<td><strong>BIP</strong> (Backward Inner Primer)</td>
<td>Composed by the B1c region at the 5' end and the B2 region at the 3' end, which is complementary to the B2c region of DNA</td>
<td>![BIP Illustration]</td>
</tr>
<tr>
<td><strong>B3 Primer</strong> (Backward Outer Primer)</td>
<td>Composed by the B3 region that is complementary to the B3c region of DNA</td>
<td>![B3 Primer Illustration]</td>
</tr>
</tbody>
</table>

The DNA polymerase used for LAMP (Bst DNA polymerase) is different from the one commonly used in PCR (Taq DNA polymerase), exhibiting strand-displacement activity. This activity allows DNA polymerase to displace dsDNA during polymerization, therefore thermal denaturation is not required and the reaction can proceed under isothermal conditions (60-65°C). The mechanism of LAMP is illustrated Figure 2.9.

The amplification starts with the invasion of the target dsDNA by one of the inner primers (FIP) and, consequently, the strand-displacement DNA polymerase extends the primer, separating the initial strands of the target DNA. The second step is initiated by the annealing of an outer primer (F3 primer) to an upstream target region, initiating the DNA synthesis and resulting in the displacement of the strand previously synthesized. This released single strand forms a loop structure at the 5' end due to the complementarity of F1c and F1 regions. In the third step, the other inner primer (BIP) anneals to its complementary region located at the 3' end of the strand and the DNA synthesis is initiated, reverting the DNA from a loop structure into a linear one. Then, the other outer primer (B3 primer) binds to its complementary region at the 3’ end and the synthesis starts, resulting in the displacement of the strand previously formed. This released single strand forms a loop structure at each end, looking like a dumbbell which serves as the starting structure for the amplification cycle of the LAMP method. This cycle is the fourth step of LAMP, where the dumbbell structure previously produced is converted into a loop structure with multiple sites for binding of primers. After DNA synthesis, a “turn over” structure of the previous dumbbell structure is displaced. This structure is used to continue the amplification cycle while the loop structure is released from the cycle. In the fifth step, the
products of the amplification cycle lead to the synthesis of various sized structures, consisting of alternately inverted repeats of the target sequence (Eiken Chemical Co., 2005).

Figure 2.9 – Schematic description of LAMP technique (adapted from (Eiken Chemical Co., 2005)).
Different methods can be used to detect the products of LAMP, such as agarose gel electrophoresis, fluorescence methods or real-time monitoring of turbidity by a turbidimeter. The turbidity derives from the precipitation of magnesium pyrophosphate, generated as a by-product that can be correlated with the amount of DNA amplified.

This method has several advantages when compared with PCR, namely the simple and cost-effective equipments required, very high amplification efficiency, higher tolerance to inhibitory materials, no need for a thermal cycler because the process is isothermal, the amplification products can be visualized directly by turbidity and there is less proneness to non-specific amplification. However, multiplexing is really challenging due to the complexity of the primers (Fakruddin, 2011). The incorporation of the LAMP method in a microfluidic setting is easier than PCR due to the multiple advantages mentioned before and it has already been demonstrated (Zanoli & Spoto, 2013).

2.5. Microscale Total Analysis System (µTAS)

Nowadays the major driving forces for developing new diagnostic and analytical techniques are reduced hands-on-time, increased sensitivity, multiplexing and faster methods. Reliable and rapid test methods are needed by both clinical laboratories and food industry. In the case of food analysis, the main objectives of having fast and reliable test are to ensure the health of consumers, to easily determine whether a food product has been subjected to cross-contamination and, simultaneously, to identify how and when this cross-contamination occurred to establish the proper corrective measures.

A lab-on-a-chip (LOC) is a device that integrates one or several laboratory functions on a single miniaturized device to achieve automation and high-throughput screening (Volpatti & Yetisen, 2014). Among LOC devices, micro Total Analysis Systems (µTAS) are dedicated to the integration of the total sequence of laboratory processes to perform an analytical procedure (Reinholt & Baeumner, 2014).

The major advantage of miniaturizing DNA analysis methods is their portability, allowing in situ detection. Moreover, these miniaturized systems are faster and more sensitive, smaller sample volumes and smaller quantities of reagents are required and they can be automated, reducing significantly the risk of contamination (Price, et al., 2009). All these characteristics come associated with a decreased cost.

An important part of any miniaturized system for DNA analysis is the DNA extraction and purification. The miniaturization of this step by itself presents a series of advantages compared to their macroscale bench-top counterparts, including decreased cost, increased speed, automation, and reduced contamination possibilities. Furthermore, miniaturization enables these technologies to be multiplexed, being able to handle multiple samples simultaneously, and provides a platform for high throughput sample processing (Reinholt & Baeumner, 2014). Other interesting advantage of miniaturized DNA extraction and purification systems, in
particular μSPE, is that it is possible to put in contact a higher volume of initial binding material with the solid phase and recover the DNA in a lower volume during the elution phase, allowing to concentrate the DNA when minute amounts of these molecules are present in the sample.

As mentioned before, the isolation of nucleic acids is crucial for the μTAS and the choice of the most adequate method must be considered according to the type of nucleic acid to be isolated, the type of sample to be analyzed and the purpose of its isolation regarding the downstream amplification and detection processes. The most common method used for amplification at microscale is PCR, but isothermal amplification techniques are becoming more popular because the integration of thermocycling into a microdevice is difficult. Another important condition is the characteristics of the device and its limitations (Reinholt & Baeumner, 2014). These and more considerations for the development of a μTAS are illustrated in Figure 2.10.

Figure 2.10 – Microdevice design considerations for the development of μTAS (Reinholt & Baeumner, 2014).
CHAPTER 3. MATERIALS AND METHODS

In order to develop and optimize a DNA extraction method from food samples using μSPE and microfluidics, this work involved both the use of commercial DNA extraction kits, previously optimized in the laboratory at INL, and the in-house development and optimization of DNA extraction protocols for their use in previously designed devices, involving μSPE on an embedded commercial silica membrane.

3.1. Commercial Kits for DNA Extraction

DNA extraction using commercial kits was performed with two different types of biological samples: sesame seeds as representative of challenging food ingredient and a solution of standard DNA (stdDNA) with known concentration (50 ng/μl). The standard DNA used was a low molecular weight DNA from salmon sperm (Sigma-Aldrich) and the commercial kits used for the extraction of these two types of samples were the NucleoSpin® Food and NucleoSpin® Plant II kits (Macherey-Nagel).

3.1.1. DNA Extraction Protocol

The protocol selected for the DNA extraction from sesame seeds was based on the process already applied and optimized in the laboratory, which resulted in the adaptation and combination of the protocols of each kit to the particular samples of interest. The altered protocol is illustrated in Figure 3.1.

This protocol starts with the homogenization of 200 mg of sesame seeds using liquid nitrogen and grinding it with a pestle and mortar. The lysis step is achieved by adding 550 μL of Buffer CF (from NucleoSpin® Food kit) pre-heated to 65°C, vortexing 15 s, adding 10 μL of Proteinase K (10 mg/ml), vortexing again 2-3 s and then incubating the mixture for 3h at 65°C with interval mix using a thermomixer (Eppendorf Thermomixer comfort). The third step is the filtration of the lysate, which is loaded onto the Filter (from NucleoSpin® Plant II kit) and centrifuged (Hettich Zentrifugen Mikro 220R) at 11000xg for 2 minutes. The volume of clear liquid in the middle of the collected flowthrough from the previous step is transferred to a new tube and equal volumes of Buffer...
C4 (from NucleoSpin® Food kit) and ethanol are added and mixed for 30 seconds in order to adjust the DNA binding conditions. After this, the binding step takes place by loading the mixture onto the column, centrifuging at 11000xg for 1 minute and discarding the flowthrough. In the washing step, the bound DNA is firstly washed with 400 μL of Buffer CQW (from NucleoSpin® Food kit) followed by 1 minute centrifugation at 11000xg. The second wash uses 700 μL of Buffer CS (from NucleoSpin® Food kit), also followed by 1 minute centrifugation at 11000xg, and the third wash uses 200 μL of the same Buffer CS, followed by 2 minutes centrifugation at 11000xg. This last centrifugation is longer in order to dry the membrane, removing residual ethanol. The last step is the elution of DNA from the membrane and it is achieved by adding 50 μL of Buffer CE (from NucleoSpin® Food kit) pre-heated to 65°C, waiting 5 minutes at room temperature and then centrifuging for 1 min at 11000xg. This elution step is repeated one more time resulting in a final volume of 100 μL of eluted DNA solution.

The protocol used for the DNA extraction from standard DNA (stdDNA) solutions is similar to the one used for the sesame seeds, however the lysis and filtration steps are not required. Thus, this protocol starts with the adjustment of the DNA binding conditions and the rest of the procedure is the same as described before.

3.2. DNA Extraction on Microfluidic Devices

The types of samples from which DNA was extracted using commercial kits were also tested using microfluidic devices. In this subchapter the design and materials composing these devices will be described, as well as the experimental set-up for DNA extraction, including the preparation of the samples and the composition of buffers used in the process.

3.2.1. Devices Design and Materials

Two washable and reusable systems were designed and fabricated in collaboration with the MEMS research group at INL. The devices were fabricated in poly (methyl methacrylate) (PMMA), also known as acrylic, which is a strong and lightweight transparent thermoplastic. The PMMA plate was cut using a computer numerical control (CNC) router (FlexiCAM), as illustrated in Figure 3.2.
The fabrication of both devices was based on the same model, which is a system with one inlet channel and one outlet channel. The fluid is introduced in the system through the inlet channel, passes through the solid-phase, which is a disposable silica membrane, and is released through the outlet channel. In order to avoid leakages, the devices were sealed with polydimethylsiloxane (PDMS), which is a silicon-based organic polymer. This polymer is a transparent and biocompatible material which can be produced with the desired pattern by using a mold, thus it is widely used in microfluidics. For these systems, two PDMS o-rings and one PDMS seal were produced with variable thickness in order to test the most adequate for this experiment. Molds for PDMS o-rings and seals were fabricated on PMMA as well. One of the o-rings is placed under the membrane and the other one is placed over it, while the seal is used to cover the region around the membrane and the channels. Therefore, the fluid is confined to the membrane region, not being able to get out through any other way besides the outlet channel. The 3D structures of both prototypes are illustrated in Figure 3.3.
**PDMS preparation:** The PDMS pre-polymer components need to be weighed in a 10:1 ratio (PDMS polymer:crosslinker) and mixed until becomes cloudy and full of bubbles. Then, the mixture is centrifuged for 6 min at 4400 rpm to remove the air bubbles. After that, the PDMS can be used immediately or kept at -20°C until further use. However, before dispensing the PDMS into the mold it must be at room temperature.

**PDMS seal and o-rings production:** The PDMS is dispensed into the mold and the excess is removed until obtaining a flat surface. The filled mold is placed into a plastic vacuum desiccator to remove the bubbles and then it is cured in the oven (Thermo Scientific™ Heratherm™) for 1h at 65°C. After the curing process the o-rings and seal can be removed from the mold.

The big difference between the two devices is their size. The prototype 1 has a central cavity to host the disposable silica membrane with a diameter of 21 mm and a volume capacity of approximately 500 μl, while the prototype 2 has a central cavity to host the disposable silica membrane with a diameter of 7 mm and a volume capacity of approximately 200 μl.

### 3.2.2. Assembly and Flow rate Tests

After the fabrication of the systems, a set of tests using dye solutions were performed in order to select the best way to assemble the devices (Figure 3.4). The prototype 1 was tested for each assembly hypothesis with a flow rate of 100 μl/min. The test was performed using o-rings with different thicknesses (0.5, 0.75 and 1 mm) to verify if it would be possible to remove the PDMS seal and use just the o-rings in order to simplify the assembly process. However it was demonstrated that the o-rings by themselves are not enough to seal the system and avoid fluid leakage, especially at high flow rates. Therefore, both o-rings and the seal are required and the best thickness for the o-rings is 1 mm.

With this assembly option, the prototypes 1 and 2 were then tested within a range of flow rates (50-500 μl/min) in order to verify if the systems could work under these conditions. This test demonstrated that the systems can support these flow rates without being observed any fluid leakage.
3.2.3. Solid-Phase Characteristics and Buffers Composition

The solid-phase used in this work was a disposable commercial silica membrane. The experiments performed with the prototype 1 always used the same type of membrane (Membrane I) but in the smaller system (prototype 2) two different types (Membranes I and II), both fabricated on borosilicate glass, were tested in order to analyze their effect on the extraction process. The Membrane I (GF/A) was cut to fit in the prototype 2. The characteristics of each type of membrane used in the experiments of this work are described in Table 3.1.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Diameter</th>
<th>Thickness</th>
<th>Particle Retention in Liquid</th>
<th>Brand</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Prototype 1 (21 mm)</td>
<td>260 μm</td>
<td>1.6 μm</td>
<td>GE Healthcare Life Sciences – Grade GF/A</td>
</tr>
<tr>
<td></td>
<td>Prototype 2 (7 mm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>Prototype 2 (7 mm)</td>
<td>675 μm</td>
<td>2.7 μm</td>
<td>GE Healthcare Life Sciences – Grade GF/D</td>
</tr>
</tbody>
</table>

The selection of the buffers used in the experiments was based on the best conditions used for DNA extraction with commercial kits and other microfluidic systems described in the literature (Breadmore, et al., 2003) (Ivanova, et al., 2008) (Vandeventer, et al., 2013). The composition of these buffers is described in Table 3.2, as well as their respective pH. As binding buffers, three chaotropic buffers (BB1, BB2 and BB3) and a non-chaotropic one (BB4) were prepared. For the washing step was used ethanol and for the elution step two elution buffers were selected: Buffer Tris-EDTA (TE) and DI water (Milli-Q). Some alternative protocols were developed using some of the binding buffers also for the washing step, by replacing completely the ethanol wash or by doing a second wash after the ethanol. All the reagents used to prepare the buffers were from Sigma-Aldrich brand.
Table 3.2 – Composition of binding, washing and elution buffers tested and compared for DNA extraction.

<table>
<thead>
<tr>
<th>Name</th>
<th>Composition</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Binding Buffers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BB1: Binding Buffer 1</td>
<td>6M GuSCN, Buffer TE (10mM Tris-HCl, 1mM EDTA), 50% EtOH</td>
<td>6.4</td>
</tr>
<tr>
<td>BB2: Binding Buffer 2</td>
<td>6M GuSCN, Buffer TE (10mM Tris-HCl, 1mM EDTA)</td>
<td>6.4</td>
</tr>
<tr>
<td>BB3: Binding Buffer 3</td>
<td>6M GuSCN, 20mM EDTA pH 8, 10mM Tris-HCl pH 6.4, 4% TritonX-100</td>
<td>7.3</td>
</tr>
<tr>
<td>BB4: Binding Buffer 4</td>
<td>0.25M Glycine, 400 mM KCl</td>
<td>5</td>
</tr>
<tr>
<td><strong>Washing Buffer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W1: Washing Buffer 1</td>
<td>ETOH 85% (v/v)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Elution Buffers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1: Elution Buffer 1</td>
<td>Buffer TE (10mM Tris-HCl, 1mM EDTA)</td>
<td>8</td>
</tr>
<tr>
<td>E2: Elution Buffer 2</td>
<td>DI water (Milli-Q)</td>
<td>-</td>
</tr>
</tbody>
</table>

3.2.4. Preparation of Biological Samples

Similarly to the experiments using the commercial kits, the biological samples tested with the microfluidic systems were a standard DNA (stdDNA) solution and sesame seeds.

For the experiments using the stdDNA solutions, the preparation of the sample before starting the extraction protocol is not required because the DNA is already in solution. Therefore, a stock stdDNA solution was prepared in Buffer Tris-Base (50mM, pH 8) with a concentration of 100 ng/μl, which was confirmed using a microvolume spectrophotometer (NanoVue™). This stdDNA solution was then mixed with the binding buffer that was going to be tested in a ratio 1:1, resulting in an initial concentration of 50 ng/μl, and introduced in the microfluidic device.

Contrarily, the sesame seeds cannot be directly introduced in the system, requiring a previous preparation to break the cell wall and lyse the cells, in order to release the DNA. This preparation starts with the lysis of the sesame seeds and the protocol depends on the binding buffer composition because some of these buffers have components that help to perform the lysis. Therefore, the binding buffers 1 and 3 can be used as lysis buffers since they include ethanol and Triton X-100 in their composition, which are able to induce this cell disruption process (Ghosal & Srivastava, 2009). For the tests with the binding buffers 1 and 3, the cell lysis is achieved by adding 4 ml of binding buffer to 1500 mg of previously grinded sesame seeds, mixing it for 60 seconds and incubating the mixture for 1h30 at 65°C with agitation. After the incubation, the lysate is centrifuged at 4000 rpm for 10 minutes and the clear liquid in the middle was collected, the pellet including the solid remains from the cell walls, together with the upper portion with low density components including fatty molecules are discarded. This aqueous solution containing the DNA can then be introduced in the system. On the other hand, for the tests with the binding buffers 2 and 4, the lysis is performed with the commercial kits by following the protocol described in Figure 3.1 until the filtration step and, then, the lysate is added to the binding buffer that
is going to be tested in a ratio 1:1. This protocol is repeated as needed in order to have the same sample volume to be introduced in the system as in the other cases, in order to be able to compare results.

3.2.5. Experimental Set-up

The prototypes were connected to a syringe pump (NE-1800 syringepump.com) with a constant flow rate for each step of DNA extraction. In each step, samples were collected for later quantification in order to analyze the variation of the amount of DNA through all the phases of this procedure. This experimental set-up is illustrated in Figure 3.5 and was applied to prototypes 1 (A) and 2 (B).

![Figure 3.5 – Experimental set-up for DNA extraction with the prototype 1 (A) and the prototype 2 (B)](image)

Different DNA extraction protocols were tested and optimized in terms of buffer composition, applicability for automation on a microfluidic setting and collected volumes for higher DNA yield. These optimized experiments will be further described in the next chapter but all of them were based on the results obtained with the initial protocol. This initial protocol was planned accordingly with the usual steps integrated in a bench scale DNA extraction process using silica membrane as described below.

Initial Protocol

As mentioned before, the DNA extraction procedure implemented in the microfluidic devices was based on the type of extraction usually performed with the commercial kits using silica membranes as solid-phase. This means that each one of the steps included in the microfluidic experiment is meant to represent the steps followed in the commercial kits. With this in mind, the initial protocol for the DNA extraction with the microdevices was defined and is schematically illustrated in Figure 3.6.
Before starting to pass the sample through the system a pre-conditioning step was performed by passing a 0.1% (v/v) Tween-20 solution (Sigma-Aldrich) followed by DI water (Milli-Q). This step allows to clean the membrane before the binding step and to remove the air initially present in the device.

The Table 3.3 summarizes the flow rates, the total volume collected and the number of collected samples in each step of DNA extraction. The flow rate for each step of the DNA extraction procedure was carefully chosen in order to facilitate all the required steps: the interaction of the DNA with the membrane during binding and the interaction of elution buffer with the membrane and DNA during elution, which required a low flow rate, as well as the washing step to remove the other components without washing down the attached DNA and the drying step to dry the membrane and eliminate the remaining ethanol.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Flow rate (μl/min)</th>
<th>Total Average Volume collected (μl)</th>
<th>Number of collected samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Prototype 1</td>
<td>Prototype 2</td>
</tr>
<tr>
<td>Binding</td>
<td>10</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>Washing</td>
<td>20</td>
<td>2000</td>
<td>1000</td>
</tr>
<tr>
<td>Drying</td>
<td>200</td>
<td>750</td>
<td>100</td>
</tr>
<tr>
<td>Elution</td>
<td>10</td>
<td>2000</td>
<td>1000</td>
</tr>
</tbody>
</table>
This initial protocol was then tested and optimized in order to obtain a protocol more suitable for integration in a microfluidic setting. Briefly, all the steps involved on the DNA extraction protocol were optimized sequentially, namely the DNA binding to the silica membrane, the washing and drying steps and elution of the DNA from the silica membrane. All these alterations and optimization tests will be further detailed in the next chapter.

3.3. Quantification and Purity of DNA

After the DNA extraction, the eluted DNA obtained with the commercial kits as well as the samples collected in each step of the extraction protocols with the microfluidic systems were quantified and the different conditions tested were compared based on those results.

3.3.1. UV-Vis Spectrophotometry

As mentioned before, the UV-Vis spectrophotometry can give information about the DNA concentration in the samples and, at the same time, about its purity. This technique was used to analyze the samples collected in the experiments with sesame seeds by using the Thermo Scientific NanoDrop 2000 spectrophotometer. This equipment is a microvolume spectrophotometer that can measure sample volumes as small as 0.5 μL. However, some of the buffers used for the DNA extraction contribute to the absorption signal, namely the chaotropic binding buffers, which may lead to an overestimation of the DNA quantity. Moreover, the presence of ssDNA and RNA also contribute for the signal. For this reason, the samples were quantified by a fluorescence method which is also a more specific and sensitive technique.

3.3.2. Fluorescence Method

The DNA quantification was achieved by using the Quant-iT™ PicoGreen® dsDNA assay kit (Invitrogen brand). PicoGreen® is a sensitive fluorescent nucleic acid stain which, when bound to dsDNA in solution, shows a very strong increase in fluorescence. This characteristic is very important because avoids the contribution of signal of ssDNA and other contaminants. The structure of this fluorescent molecule and a model for PicoGreen®/dsDNA complex formation proposed by Dragan et al. is represented in Figure 3.7 (Dragan, et al., 2010).
The fluorospectrometer used to quantify the samples with PicoGreen® reagent, was the Thermo Scientific NanoDrop 3300, which has the ability to measure as little as 1 μl of sample and, therefore, only uses a fraction of sample commonly needed for conventional cuvette-based fluorometers. This ability allows the detection of significantly scaled-down volumes in the extraction process, which is crucial for the miniaturization of this technique.

The quantification experiments used an excitation maximum of 470 nm and an emission maximum of 525 nm. In order to correlate the emitted fluorescence with the dsDNA concentration in the samples, a standard curve was required. This curve was obtained by measuring serially diluted dsDNA standards with the following final concentrations: 0, 1, 5, 10, 25, 100, 500 and 1000 ng/ml. These standards were diluted with 1x TE Buffer, which was also used as a blank for the measurements. The concentrated PicoGreen® dye stock was diluted two hundred fold and used as working solution. All the standard dilutions and samples measured had a sample to working solution ratio of 1:1.

### 3.4. Efficiency of DNA Extraction

The tests performed with known concentration stdDNA solutions were used to evaluate the efficiency of the DNA extraction processes. This efficiency can be determined by using the equation (3.1).

\[
\text{Efficiency of DNA Extraction (\%)} = \frac{\text{Amount of DNA recovered in the Elution step}}{\text{Amount of DNA initially present in the sample}} \times 100 \tag{3.1}
\]

The DNA extraction efficiency can be used to compare different extraction protocols tested in the same system as well as different extraction systems. For example, conventional and microfluidic techniques based on the same type of extraction can be compared using this parameter.
CHAPTER 4. RESULTS AND DISCUSSION

In this chapter, the results of the DNA extraction from two different samples (standard DNA solution and sesame seeds) using both commercial kits and microfluidic systems will be presented, as well as the tests performed for optimization of the protocol used in the microdevices.

4.1. Commercial Kits for DNA Extraction

Using the protocol described in Figure 3.1 from the previous chapter, the commercial kits were used to extract DNA from a stdDNA solution and from sesame seeds. The initial concentration of the stdDNA solution tested was 50 ng/μl.

4.1.1. Standard DNA Solution

The DNA extraction from the standard solution was executed for determination of the extraction yield obtained with this technique, using the equation (3.1) presented in the previous chapter.

All the experiments for DNA extraction from the standard solution using the kits were done in triplicate in order to verify if there was technical reproducibility and to minimize the errors when comparing different methods of extraction. The results obtained with this sample are presented in Table 4.1.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Initial Sample</th>
<th>Eluted Sample</th>
<th>Efficiency of DNA Extraction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Concentration (ng/ml)</td>
<td>1075±171</td>
<td>394±36</td>
<td>10.5</td>
</tr>
<tr>
<td>Volume (ml)</td>
<td>0.35</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>Mass (ng)</td>
<td>376</td>
<td>39.4</td>
<td></td>
</tr>
</tbody>
</table>

As mentioned before, the fluorescent method used for DNA quantification was performed using the Quant-iT™ PicoGreen® dsDNA assay kit. PicoGreen® binds to the dsDNA in solution emitting strong fluorescence, however, it is important to refer that the reagent available in the laboratory was not reacting as estimated. Consequently, the intensity of the signal was much lower than expected and, therefore, the initial average concentration was underestimated. The stdDNA solution with an initial concentration of 50 ng/μl was diluted by a factor of 100 in order to obtain a value inside the range of concentrations used to determine the standard curve (0-1000 ng/ml). As a result, the diluted stdDNA solution (500 ng/ml) was measured with the PicoGreen® reagent and the average concentration obtained was 10.75 ng/ml. After correction with the dilution factor, the initial concentration determined was 1075 ng/ml, being underestimated. Since this technique was the one selected to quantify the samples, this last value was used to determine the extraction
yields of all the experiments using the stdDNA solution. This approximation was made considering that the error associated with the reactivity of PicoGreen® would be the same in all the measurements, which means that, although the concentration value measured is not the real one, the calculated yields would be the same and it also allows a qualitative comparison between the experiments.

4.1.2. Food Sample: Sesame Seeds

The extraction of DNA from sesame seeds was performed in order to compare the results from the commercial kits with the results obtained with the fabricated microfluidic systems using a complex sample. The DNA extraction from the sesame seeds was also done in triplicate. Since this sample is a food matrix it has much more compounds compared with the stdDNA solution, so the UV-Vis spectrophotometer (Nanodrop 2000) was used to analyze the purity of the eluted DNA after the extraction with the kits. The results obtained with this technique are summarized in Table 4.2. The volume of sample resulting from the lysis step was approximately 350 μl.

<table>
<thead>
<tr>
<th>Food Sample</th>
<th>Concentration (ng/μl)</th>
<th>Concentration (ng/ml)</th>
<th>Purity (A260/A280)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sesame A</td>
<td>269.1</td>
<td>269100</td>
<td>1.75</td>
</tr>
<tr>
<td>Sesame B</td>
<td>79.50</td>
<td>79500</td>
<td>1.86</td>
</tr>
<tr>
<td>Sesame C</td>
<td>212.4</td>
<td>212400</td>
<td>2.07</td>
</tr>
</tbody>
</table>

The results obtained regarding the purity of the eluted DNA were very good, since a DNA sample can be considered “pure” if its ratio A260/A280 is between 1.7 and 2.0. The sesame seeds used as sample in these experiments were all from the same batch and the extraction process was performed at the same time and with the same conditions. Thus the variability observed in the concentration results may be related with unavoidable random errors during both the lysis step and DNA purification procedures. The grinded seeds were not exactly the same size and that can affect the lysis efficiency because smaller particles are easier to lyse than larger ones. Smaller particles have higher surface area for contact with the lysis buffer, being more readily disrupted and dissolved into solution. Moreover, this variation was not so evident when extracting DNA from the standard solution, which does not require a lysis step.

In order to compare the results of DNA extraction from sesame seeds using commercial kits with the results obtained using microfluidic systems, the DNA concentration from the samples was also quantified by using the Quant-it™ PicoGreen® dsDNA assay kit. The results obtained with this technique are presented in Table 4.3.
Table 4.3 – Results of DNA extraction from sesame seeds using commercial kits obtained with PicoGreen®.

<table>
<thead>
<tr>
<th>Food Sample</th>
<th>Sesame Seeds Mass (mg)</th>
<th>Concentration (ng/ml)</th>
<th>Final Volume (ml)</th>
<th>Final DNA mass (ng)</th>
<th>Ratio (ng DNA/mg sesame seeds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sesame A</td>
<td>200</td>
<td>898.0</td>
<td>0.1</td>
<td>89.80</td>
<td>0.45</td>
</tr>
<tr>
<td>Sesame B</td>
<td>200</td>
<td>513.7</td>
<td>0.1</td>
<td>51.37</td>
<td>0.26</td>
</tr>
<tr>
<td>Sesame C</td>
<td>200</td>
<td>935.4</td>
<td>0.1</td>
<td>93.54</td>
<td>0.47</td>
</tr>
</tbody>
</table>

Since the quantity of sesame seeds used as a sample for DNA extraction with these kits and with the microfluidic systems was not the same, the ratio of extracted DNA to sesame seeds mass was determined and can be used for comparison of results. In the commercial kits, were used approximately 200 mg of sesame seeds for each experiment while in the microfluidic tests the amount of sesame seeds used for extraction varied between approximately 550 mg and 1500 mg depending on the protocol used for the preparation of samples, as described in the previous chapter.

The differences between the DNA concentration estimated by the UV-Vis spectrophotometer and the fluorometer might be explained by the fact that PicoGreen® binds to dsDNA in solution and avoids the contribution of signal of ssDNA and other contaminants. Moreover, the DNA concentration results obtained with PicoGreen® are underestimated as mentioned before.

4.2. Microfluidic System: Prototype 1

The prototype 1 was also tested using the standard DNA solution and sesame seeds. The stdDNA solution (1075 ng/ml) was used to test the initial protocol, described in Figure 3.6 from the previous chapter, and for its optimization. Several tests were performed in order to develop and optimize a protocol suitable for automation in a microfluidic setting and with high DNA recovery from samples. The results were compared by determination of the DNA extraction yields. After optimization, this prototype was tested using sesame seeds as an example of challenging food sample for DNA extraction and the results were compared with the initial protocol.

4.2.1. Optimization of Binding Step

Standard DNA solutions were used to optimize each step of DNA extraction starting with the previously described Initial Protocol. The first experiments were performed for the optimization of the binding step and different chaotropic binding buffers (BB1, BB2 and BB3), as described in Table 3.2, were tested. Chaotropic buffers are normally used because they facilitate the DNA binding to silica surfaces when compared with non-chaotropic buffers. These three different binding buffers were tested in order to select the one with the best binding efficiency.
This protocol started with the preparation of the sample to be analyzed by mixing the binding buffer to be
tested with the stock stdDNA solution in a ratio 1:1. After passing the sample through the system, the
previously described washing step was performed in order to remove the unbound contaminants, including
unbound DNA, and, then, dried by passing air through the system to remove the remaining ethanol from the
washing step. The elution step took place after that and the elution buffer 1 (E1) was selected to test this
protocol. The composition of all the buffers used as well as the flow rates selected and the buffer volumes
passed in each step are listed in the Table 3.2 and Table 3.3 from the previous chapter, respectively.

The tests using this initial protocol were performed in triplicate and samples were collected in each step
and quantified with PicoGreen®. This quantification showed the profile of DNA extraction using the three
different chaotropic binding buffers and the results are presented in Figure 4.1.

![Figure 4.1 – Test of Initial Protocol using chaotropic binding buffers (BB1, BB2 and BB3) in prototype 1.](image)

Ideally, the DNA extraction profile should show none or very low loss of DNA during the binding, washing
and drying steps and high amounts of DNA collected in the elution step. According to Figure 4.1, although some
DNA was lost in the binding step, low amounts of DNA were released during the washing and drying steps and,
consequently, the phase in which most DNA was collected was the elution step as expected. The BB3 had the
best binding results since lower amounts of DNA were lost in this phase, however, the best elution results were
obtained with BB1, which had the best elution efficiency as presented in Table 4.4.

<table>
<thead>
<tr>
<th>Binding Buffer Tested</th>
<th>Elution Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BB1</td>
<td>10.3</td>
</tr>
<tr>
<td>BB2</td>
<td>8.9</td>
</tr>
<tr>
<td>BB3</td>
<td>9.4</td>
</tr>
</tbody>
</table>

It is important to refer that some variability was observed between the triplicates, resulting in high standard
deviation, especially in the drying and elution steps, which may be related with the presence of air bubbles in
the system. The drying step is needed in this protocol to remove the ethanol remaining from the washing step because it can inhibit the downstream amplification. However, the presence of air bubbles during the elution step affects the efficiency of DNA extraction since it may reduce the membrane surface area contacting with the elution buffer. Moreover, it brings a limitation for the integration of extraction and amplification processes in a microfluidic setting.

4.2.2. Optimization of Washing Step

Considering the limitations that the presence of air bubbles in the microfluidic systems bring to DNA analysis procedures, a set of alternative protocols were tested in order to remove the drying step performed after the washing step.

4.2.2.1 Test of Alternative Protocols

Four alternative protocols were developed in order to overcome the limitations demonstrated with the initial one. These alternatives were essentially focused in the alteration of the washing step, either by the substitution of ethanol as a washing buffer or by the addition of a second wash to remove the remaining ethanol from the first one. The main steps included in these new approaches are described in Table 4.5.

<table>
<thead>
<tr>
<th>Alternative Protocol</th>
<th>Binding Step</th>
<th>Washing Step</th>
<th>Elution Step</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Chaotropic Binding Buffers (BB2)</td>
<td>Binding Buffer (BB2)</td>
<td>Elution Buffer 1</td>
</tr>
<tr>
<td>II</td>
<td>Chaotropic Binding Buffers (BB2)</td>
<td>1st Wash: Ethanol 85% (v/v) 2nd Wash: Binding Buffer (BB2)</td>
<td>Elution Buffer 1</td>
</tr>
<tr>
<td>III</td>
<td>Chaotropic Binding Buffers (BB1, BB2, BB3)</td>
<td>1st Wash: Ethanol 85% (v/v) 2nd Wash: Non-Chaotropic Binding Buffer (BB4)</td>
<td>Elution Buffer 1</td>
</tr>
<tr>
<td>IV</td>
<td>Non-Chaotropic Binding Buffer (BB4)</td>
<td>Binding Buffer (BB4)</td>
<td>Elution Buffer 1</td>
</tr>
</tbody>
</table>

**Alternative Protocol I**

The first alternative tested was based on using the binding buffer not only for the binding step but also for the washing step. So, after the binding step the unbound contaminants were removed by passing approximately 2000 μl of binding buffer (without DNA) through the system instead of ethanol. This way there is no need for a drying step because this protocol does not use ethanol as a washing buffer. This alternative was
tested using one of the chaotropic binding buffers used in initial protocol, namely BB2. The results obtained with this approach after quantification with PicoGreen® are illustrated in Figure 4.2.

![Figure 4.2 - Test of Alternative Protocol I using a chaotropic binding buffer (BB2).](image)

The quantification results demonstrated that this approach is not a good alternative because, although the protocol was easier to perform due to the removal of the drying step, no elution was observed.

**Alternative Protocol II**

The second alternative protocol tested consisted in performing two washing steps after the DNA binding: one using 2000 μl of ethanol and a second one using 1500 μl of binding buffer (without DNA). The second wash is used to remove the ethanol introduced in the first wash and, consequently, substitutes the drying step. This approach was tested using the same chaotropic binding buffer used in the test with alternative protocol I (BB2). The results obtained using this alternative after quantification are illustrated in Figure 4.3.

![Figure 4.3 – Test of Alternative Protocol II using a chaotropic binding buffer (BB2).](image)
Similarly to the test with the alternative protocol I, the results obtained with this second approach demonstrated that this was also not a good alternative because, once again, no elution was observed.

The results of these two alternatives may be explained by the fact that the binding buffer used has a high concentration of a strong chaotropic salt (guanidine thiocyanate, GuSCN). Since this buffer is also used in the washing step, when the elution occurs there is still too much salt present in the system and the conditions for elution of the bound DNA are not favorable. Consequently, the DNA remains bound to the membrane instead of being collected during the elution step.

With this in mind, a non-chaotropic buffer (BB4) was prepared and tested not only as a binding buffer but also as a washing buffer, resulting in the development of the third and fourth alternative protocols.

**Alternative Protocol III**

The third alternative protocol also included two washing steps. The first wash was performed by passing 2000 μl of ethanol while the second one was done by passing 1500 μl of non-chaotropic buffer (BB4) through the system. The main goal of using this non-chaotropic buffer instead of a chaotropic one, like happened in the alternative protocol II, was to be able to remove the ethanol remaining from the first wash but, at the same time, to have more favorable conditions for the following step, which is the elution of DNA. The binding of DNA to the silica membrane is not as strong in non-chaotropic conditions as it is with chaotropic buffers. Thus, this new buffer should be able to remove the ethanol without limiting the elution because its ionic strength is lower than the ionic strength of chaotropic buffers. However, the conditions of the second wash must still be good enough to not remove the DNA bound to the membrane, which means that the ionic strength needs to be high enough and the pH should favor the adsorption of DNA to the membrane, otherwise the DNA would be lost during the second wash.

Like in the previous alternatives, a chaotropic buffer (BB2) was used for the binding step. This test was also done in triplicate and the results obtained are presented in Figure 4.4.

![Figure 4.4 - Test of Alternative Protocol III using a chaotropic binding buffer (BB2).](image-url)
With this third approach, some DNA was collected during the elution step, which supports the hypothesis previously used to explain the poor elution efficiency obtained with the alternative protocols I and II. However, it was observed that too much DNA is lost during the second wash, which should be optimized.

**Alternative Protocol IV**

The fourth and last alternative protocol tested was based on the first alternative approach but, instead of using a chaotropic buffer, a non-chaotropic binding buffer (BB4) was used. Therefore, this non-chaotropic buffer was applied not only for the binding step but also for the washing step.

The results obtained with this alternative protocol after quantification are presented in Figure 4.5.

![Figure 4.5 - Test of Alternative Protocol IV using a non-chaotropic binding buffer (BB4).](image)

With this approach, the amount of DNA collected during the elution step increased significantly and, like alternative III, the absence of a drying step makes this protocol much easier to apply in a microfluidic setting because air bubbles are avoided and, consequently, the variability among the triplicates is much lower. However, too much DNA was lost during the binding and washing steps, leaving room for optimization.

The elution efficiencies determined for each alternative protocol are presented in Table 4.6.

<table>
<thead>
<tr>
<th>Alternative Protocol</th>
<th>Elution Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>4.4</td>
</tr>
<tr>
<td>IV</td>
<td>14.0</td>
</tr>
</tbody>
</table>
The elution efficiency obtained with the alternative protocol IV was a bit higher than the efficiencies achieved with the initial one, which makes this approach very promising as a protocol suitable for DNA extraction with microfluidic systems. This alternative avoids the use of PCR inhibitors like ethanol and chaotropic salts as well as the need for a drying step that would introduce air bubbles into the system and limit its integration in a total analysis system approach.

4.2.2.2 Combined Optimization of Binding and Washing Steps

The alternative protocols with the best results and, consequently, the most appropriate to replace the initial protocol were the alternatives III and IV. However, these approaches can still be improved, especially regarding the binding and washing steps, in which high amounts of DNA were lost. Therefore, some tests were executed in order to optimize these two approaches.

Optimization of Alternative Protocol III

For optimization of alternative protocol III, the volume of both washing steps was reduced in order to decrease the amount of DNA lost during this phase. However it is important to find a balance between the volume of washing buffer and its ability to remove the contaminants, because if the volume introduced is not enough to wash out the contaminants the DNA collected in the elution step will not be so pure.

Considering that the prototype 1 has a volume capacity of approximately 500 μl, the volume of buffer used in the first wash (Ethanol) was reduced from 2000 to 1000 μl while the volume of buffer used in the second wash (BB4) was reduced from 1500 to 1000 μl. This test was performed using BB2 as binding buffer and the results obtained are illustrated in Figure 4.6.

![Figure 4.6 – Optimization of washing steps in alternative protocol III through the reduction of washing volume.](image-url)
By reducing the volume of buffer in the washing steps the amount of DNA lost in this phase was highly decreased and, consequently, the amount of DNA collected during the elution step was higher, improving the efficiency.

As mentioned before, this optimization was achieved using BB2 as binding buffer but this optimized protocol was then tested with the other two chaotropic binding buffers (BB1 and BB3) in order to choose the one with best results. The results of this test are presented in Figure 4.7.

![Figure 4.7 – Test with chaotropic binding buffers (BB1, BB2 and BB3) using alternative protocol III after optimization.](image)

According with the obtained results, the best binding buffer was BB3 because it was the one that resulted in less loss of DNA during the binding and washing steps as well as in higher elution efficiency. The extraction efficiencies determined for each test with alternative protocol III are summarized in Table 4.7.

**Table 4.7 - Elution Efficiency of alternative protocol III before and after optimization of binding and washing steps.**

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Elution Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternative Protocol III (using BB2 as binding buffer)</td>
<td>4.4</td>
</tr>
<tr>
<td>Alternative Protocol III optimized (using BB1 as binding buffer)</td>
<td>5.8</td>
</tr>
<tr>
<td>Alternative Protocol III optimized (using BB2 as binding buffer)</td>
<td>6.6</td>
</tr>
<tr>
<td>Alternative Protocol III optimized (using BB3 as binding buffer)</td>
<td>8.9</td>
</tr>
</tbody>
</table>

After optimization of binding and washing steps, the efficiency of DNA extraction using the alternative protocol III was improved, increasing from 4.4 to 8.9%, which is similar to the yields obtained with the initial protocol.
Optimization of Alternative Protocol IV

Like in the optimization of the third alternative protocol, the optimization of the washing step in the fourth approach was achieved by reducing the volume of buffer used in this step. The buffer used to wash the unbound components was the non-chaotropic buffer (BB4) and the volume passed through the membrane in this step was reduced from 2000 μl to 1000 μl. The results obtained with this optimization are illustrated in Figure 4.8.

According with the quantification results, the amount of DNA lost during the binding step using this protocol was relatively high, being approximately 6 times the amount lost in the same step using chaotropic binding buffers. For this reason, a pre-treatment of the membrane was performed in order to enhance the binding of DNA to silica. This pre-treatment consisted in passing approximately 2500 μl of non-chaotropic binding buffer (BB4) without DNA through the membrane before the binding step. The results obtained with this optimization are also illustrated in Figure 4.8.

The results demonstrated that the reduction of washing volume decreases the amount of DNA lost during this step and, consequently, better elution efficiency was obtained. In addition, the membrane pre-treatment resulted in a big reduction of the amount of DNA lost during the binding step.

The buffer used for the membrane pre-treatment was the non-chaotropic buffer (BB4), which is an amino acid buffer. The amino acid included in its composition was glycine, which is the smallest of the 20 amino acids commonly found in proteins. The amount of DNA bound to the silica membrane increased by passing this buffer through the system before the binding step and, although the molecular mechanism is not yet fully clarified, previous studies showed that this mechanism might involve interaction of the amino acid buffer with both DNA and silica membrane, depending on pH conditions (Vandeventer, et al., 2013). For lower pH conditions, the negatively charged carboxylate group of glycine interacts with the silica membrane, while the positively charged amine group is exposed. This configuration facilitates the interaction between the negatively
charged DNA and the positively charged group of glycine, contributing for a more efficient binding step. For higher pH conditions, the silica surface becomes more negatively charged favoring the interaction with the amine group of glycine, therefore, the negatively charged carboxylate group is exposed and the interaction with the DNA is no longer favorable, resulting in DNA elution.

Therefore, the combination of the washing volume reduction with the membrane pre-treatment improved the elution efficiency significantly, increasing from 14% to 44%. The DNA extraction efficiencies determined for the optimization tests performed with the alternative protocol IV are summarized in Table 4.8.

Table 4.8 - Elution Efficiency of alternative protocol IV before and after optimization of binding and washing steps.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Elution Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternative Protocol IV</td>
<td>14.0</td>
</tr>
<tr>
<td>Alternative Protocol IV (with reduced washing volume)</td>
<td>35.0</td>
</tr>
<tr>
<td>Alternative Protocol IV (with reduced washing volume and membrane pre-treatment)</td>
<td>44.0</td>
</tr>
</tbody>
</table>

4.2.3 Optimization of Elution Step

A set of different elution conditions was tested using the Alternative Protocol IV after optimization of binding and washing steps. The first elution condition (Elution I) was the one performed in the previous steps, which uses the Buffer TE as elution buffer (Elution Buffer 1) and is executed at room temperature. The second elution test (Elution II) was performed in the same conditions as Elution I but using another elution buffer, namely DI water (Elution Buffer 2). In order to analyze the influence of incubation time and temperature in the elution step, two more conditions were tested. The third elution test (Elution III) was performed using the elution buffer 1 with 5 minutes of incubation time at room temperature while the fourth and last test (Elution IV) was performed in the same conditions as Elution III but with a temperature of approximately 63°C. All the conditions tested for this optimization are listed in Table 4.9.

Table 4.9 – Elution tests using Alternative Protocol IV after optimization of binding and washing steps.

<table>
<thead>
<tr>
<th>Elution</th>
<th>Buffer</th>
<th>Temperature</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Elution Buffer 1</td>
<td>Room Temperature</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>Elution Buffer 2</td>
<td>Room Temperature</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>Elution Buffer 1</td>
<td>Room Temperature</td>
<td>5 minutes</td>
</tr>
<tr>
<td>IV</td>
<td>Elution Buffer 1</td>
<td>63°C</td>
<td>5 minutes</td>
</tr>
</tbody>
</table>
The incubation time was considered as the time during which the system was stopped between each collected sample in the elution step. For Elution IV the temperature was increased by putting the systems in a sand bath (Harry-Gestigkeit) and controlling it with a thermometer as illustrated in Figure 4.9.

![Experimental set up for tests using Elution IV with prototype 1.](image)

The results obtained after quantification with PicoGreen® are presented in Figure 4.10.

![Optimization of elution step using Alternative Protocol IV after optimization of binding and washing steps.](image)

According to the obtained results, the best efficiency was achieved by the conditions used in Elution IV, which include 5 minutes of incubation and a temperature of 63°C. This result supports previous studies in which was demonstrated that higher incubation time and temperature during the elution step improved the efficiency of DNA extraction (Huang, et al., 2009) (Ji, et al., 2007).

When comparing the tests with Elution I and III, which only difference is the 5 minutes of incubation time in the Elution III, the quantification results do not support the previous conclusions. The elution efficiency
determined for Elution III (with 5 minutes incubation) seems to be lower than the one obtained for Elution I (without incubation). This unexpected result may be associated with an unavoidable random error during the experiment, related with the buffers introduced in the system, or during the quantification procedure for the Elution III test. For future work this elution test should be repeated in order to verify the result.

The tests with Elution I and II were performed in the same conditions but using different elution buffers. The comparison of these two tests gives information about the best elution buffer, which was the Elution Buffer 1 (Buffer TE) since the amount of eluted DNA was higher than using the Elution Buffer 2 (DI water). However, both buffers demonstrated good results. The extraction efficiencies determined for each elution test are summarized in Table 4.10.

Table 4.10 - Efficiencies of each elution test performed using the alternative protocol IV after optimization of binding and washing steps

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Elution Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elution I</td>
<td>44.0</td>
</tr>
<tr>
<td>Elution II</td>
<td>32.6</td>
</tr>
<tr>
<td>Elution III</td>
<td>20.2</td>
</tr>
<tr>
<td>Elution IV</td>
<td>45.8</td>
</tr>
</tbody>
</table>

The DNA extraction efficiency obtained with the Alternative Protocol IV after optimization of all steps was 45.8 %, which is much higher than the yield obtained with the initial protocol or with the commercial kits.

4.2.4 Comparison of DNA Extraction Protocols

The results obtained with the Initial Protocol and with the Alternative Protocol IV before and after optimization are illustrated in Figure 4.11. The drying step represented in this figure is part of the initial protocol but is not included in the alternative one.
The integration of these results on the same graph allows an easier comparison between the initial protocol and the optimized one, as well as between the use of chaotropic and non-chaotropic binding buffers.

Comparing the chaotropic binding buffers (BB1, BB2 and BB3) used in the Initial protocol with the non-chaotropic one (BB4) used in the Alternative Protocol IV, it was demonstrated that the adsorption of DNA to the silica membrane is much stronger with chaotropic buffers. However, the amount of DNA eluted using the Alternative IV is similar to the amount eluted using the Initial Protocol. This result suggests that, although chaotropic buffers allow a more efficient binding of DNA to the membrane, the DNA elution is then more difficult to achieve as suggested by Vandeventer et al. (Vandeventer, et al., 2013).

With the optimization of Alternative Protocol IV, the amount of DNA collected in the elution step was significantly improved when compared with the other tests. Moreover, this approach does not need a drying step, which is a big advantage for the integration of DNA extraction in microfluidic devices, and this alternative protocol does not use chaotropic salts or ethanol, which could inhibit the downstream amplification.

4.2.3. Food Sample: Sesame Seeds

After testing and optimizing the extraction protocol using the stdDNA solution, the prototype 1 was used to extract DNA from sesame seeds.

Firstly, the DNA was extracted from the sesame seeds using the initial protocol with the chaotropic binding buffers (BB1, BB2 and BB3). The preparation of the samples depends on the binding buffer composition as described in the previous chapter. The extraction results obtained after quantification using PicoGreen® are illustrated in Figure 4.12.

![Figure 4.12 – DNA extraction from sesame seeds using the Initial Protocol with chaotropic binding buffers.](image-url)
The best results of DNA extraction from sesame seeds with the initial protocol were obtained using the buffer BB2 for the binding step since more DNA was collected in the elution step and less was lost in the previous steps. However, it is important to consider the differences in the preparation of the samples because for the tests with BB1 and BB3 the lysis was performed by incubation with the binding buffer while for the test with BB2 the lysis was done using the commercial kits.

It was observed that, in the tests of BB1 and BB3, there was formation of a precipitate during the binding step due to the high concentration of chaotropic salts. This precipitation was not observed in the test with BB2, what could be explained by the addition of proteinase K in its lysis protocol, which is an enzyme that digests proteins. It is known that chaotropic agents are cosolutes that can disrupt the hydrogen bonding network between water molecules and reduce the stability of the native state of proteins by weakening the hydrophobic effect (Salvi, et al., 2005), and therefore might promote the precipitation of proteins, which will explain the precipitate observed for BB1 and BB3. Moreover, these binding buffers have other compounds in its composition that may contribute for this precipitation, such as ethanol. The presence of precipitate in the system makes more difficult to bind the DNA to the membrane and it also contributes for the variability observed between the triplicates, resulting in big standard deviation. Therefore, the results from the tests with BB1 and BB3 were inconclusive and, in future experiments, should be tested using a lysis method similar to the one used in the test with BB2.

The Alternative Protocol IV previously optimized was also used to extract DNA from sesame seeds with this prototype. The lysis method used for this protocol was the same as the one used for the initial one with BB2, so the results obtained from these two methods are comparable and are illustrated in Figure 4.13.

![Figure 4.13 – DNA extraction from sesame seeds using the Initial Protocol with chaotropic binding buffer BB2 and using the Alternative Protocol IV after optimization.](image)

The amount of DNA eluted using the initial protocol was higher than with the Alternative IV optimized, however high variability was observed in this step, resulting in a big standard deviation. Since no precipitation was observed, this high variability of the initial protocol may be related with the presence of air bubbles during
elution, which were introduced with the drying step. As mentioned before, the Alternative Protocol IV does not need a drying step because an ethanol-free wash is used, therefore the variability between the triplicates is much lower and, consequently, the reproducibility of results is enhanced. Moreover, the alternative protocol is more suitable for a microfluidic set up and for integration of further steps of DNA analysis.

These results do not corroborate the results obtained using stdDNA solutions as a sample however it is important to consider that those solutions and sesame seeds are two very distinct matrices. When extracting DNA from a plant or food sample, even a simple one like sesame seeds, there are many other constituents present in the lysate besides DNA. The presence of these other components can interfere with the DNA extraction process, for example they may compete with the DNA during the binding step. In addition, the quality of DNA obtained from the lysis of sesame seeds is not the same as the quality of DNA present in standard solutions, which may also contribute for the differences observed between the two types of sample.

The quantification results of DNA elution using PicoGreen® protocol on the fluorospectrometer are summarized in Table 4.11, as well as the purity results obtained using the UV-Vis spectrophotometer.

<table>
<thead>
<tr>
<th>Food Sample</th>
<th>Sesame Seeds Mass (mg)</th>
<th>Final Mass of eluted DNA (ng)</th>
<th>Ratio (ng DNA/mg sesame seeds)</th>
<th>Purity (A260/A280)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysate from Sesame seeds (Initial Protocol with BB2)</td>
<td>550</td>
<td>392.0</td>
<td>0.71</td>
<td>1.05</td>
</tr>
<tr>
<td>Lysate from Sesame seeds (Alternative Protocol IV after optimization)</td>
<td>550</td>
<td>128.8</td>
<td>0.23</td>
<td>1.12</td>
</tr>
</tbody>
</table>

The ratio of DNA mass (ng) to sesame seeds mass (mg) determined with the results obtained using the initial protocol was higher than with the Alternative Protocol IV after optimization, which was expected regarding the quantification results illustrated before. However, the elution results obtained with the initial protocol had a high standard deviation and, consequently, the ratio determined for this protocol has also a big error associated.

The ratios of DNA to sesame seeds obtained using the commercial kits were higher than the one determined with the results of Alternative Protocol IV and the purity results were also better with the kits. The absorbance ratios A260/A280 measured in the samples from the extraction with prototype 1 indicate considerable protein contamination. Therefore, more tests using sesame seeds as a sample are required in order to improve the purity of the extracted DNA using this microdevice.
4.3. Microfluidic System: Prototype 2

The prototype 2 was developed as a miniaturization of the prototype 1 being based on the same DNA extraction principles. This miniaturization brings several advantages, namely the reduction of sample and buffer volumes required and the ability to perform the DNA extraction more rapidly. With the prototype 1, promising results were achieved, however the time consumed in the process was long. The extraction with the prototype 1 using the initial protocol took approximately 8 hours and, even after optimization, it took approximately 6 hours. These estimations were obtained without considering the time required to assemble the systems and to change the buffers in the syringes. Although the extraction time was reduced by optimization, it still requires a long time, which could be reduced even more by using a smaller device (prototype 2).

With the prototype 2 only the stdDNA solution was tested. Firstly the initial protocol was performed in order to verify if this system could overcome some of the problems observed with the prototype 1. After that, the two best alternative protocols were also tested in this small device and optimized according to the obtained results. Finally, two types of silica membranes were used to analyze their influence on the DNA extraction process.

4.3.1. Test of Initial Protocol

Considering that this device is much smaller than the prototype 1, the volumes collected as samples in each step of the extraction were reduced. The volume collected in each sample for quantification was 250 μl and all tests were performed in triplicate in order to determine the reproducibility of the results.

The Initial Protocol was tested using the chaotropic binding buffers (BB1, BB2 and BB3) and the results obtained after quantification with PicoGreen® are illustrated in Figure 4.14.

Figure 4.14 - Test of Initial Protocol using chaotropic binding buffers (BB1, BB2 and BB3) in prototype 2.
Using the initial protocol, the chaotropic buffer BB2 seems to have the best results in every step of the extraction and, consequently, the best elution efficiency (Table 4.12). However, the DNA yields were very low and the reproducibility of results was not very good due to the presence of air bubbles. The presence of air inside the system had a bigger effect on the prototype 2 than on prototype 1, because in the smaller device (prototype 2) the bubbles covered the entire membrane surface while, in the bigger one (prototype 1), the membrane was not completely covered, thus considerably reducing the surface interaction between the solutions and the membrane. Moreover, the air bubbles present at the beginning of the experiment were harder to remove in the prototype 2, requiring a higher flow rate.

Table 4.12 – Elution efficiency obtained using the Initial Protocol with the chaotropic binding buffers in prototype 2.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Elution Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Protocol with BB1</td>
<td>1.17</td>
</tr>
<tr>
<td>Initial Protocol with BB2</td>
<td>1.61</td>
</tr>
<tr>
<td>Initial Protocol with BB3</td>
<td>0.91</td>
</tr>
</tbody>
</table>

4.3.2. Test of Optimized Alternative Protocol

Since the problems related with the Initial Protocol were aggravated in the prototype 2, the two best alternative protocols (without drying step) were also tested in order to analyze the results without the influence of air bubbles.

Alternative Protocol IV

The first alternative protocol tested was the fourth one because it had the best results in the prototype 1. The conditions selected for elution in this test were the ones described for Elution I and the results obtained after quantification are illustrated in Figure 4.15.
With this approach the results obtained were reproducible but the efficiency of DNA elution was really low (0.47 %) especially considering the amount of DNA initially introduced in the system. Since this device is much smaller, it was consider the possibility of having DNA too diluted in the collected samples because the volumes collected were higher than the volume capacity of the device. Since the DNA is majorly eluted in the first volume fractions of elution step, the following collected volumes may contribute to dilute the sample and, consequently, the collected DNA may not be detectable with the quantification techniques. In order to better understand what happens in each extraction step, especially during elution, and considering the volume capacity of the prototype 2 (200 μl), the volumes of the initial sample and buffers introduced in the procedure were reduced according with the information in Table 4.13, being collected in each step several samples with volumes of approximately 100 μl instead of 250 μl.

Table 4.13 – Flow rate, total volume and number of samples collected in each step of DNA extraction with prototype 2.

<table>
<thead>
<tr>
<th>Extraction Step</th>
<th>Flow rate (μl/min)</th>
<th>Total Volume collected (μl)</th>
<th>Number of collected samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding</td>
<td>10</td>
<td>500</td>
<td>5</td>
</tr>
<tr>
<td>Washing</td>
<td>20</td>
<td>500</td>
<td>5</td>
</tr>
<tr>
<td>Elution</td>
<td>10</td>
<td>500</td>
<td>5</td>
</tr>
</tbody>
</table>

With these alterations in the protocol, the prototype 2 was tested again and the results of quantification are presented in Figure 4.16.

The results obtained after the protocol alterations were slightly better regarding the elution efficiency, which is presented in Table 4.14. The amount of eluted DNA compared with the amount of DNA introduced with the initial sample was improved by reducing the volumes of the collected samples however the results were still too low.
Table 4.14 – Elution efficiency obtained using the Alternative IV, previously optimized, before and after the protocol modifications in prototype 2.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Elution Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternative Protocol IV</td>
<td>0.47</td>
</tr>
<tr>
<td>(optimized with prototype 1 using Elution I)</td>
<td></td>
</tr>
<tr>
<td>Alternative Protocol IV with less volume</td>
<td>1.6</td>
</tr>
<tr>
<td>(optimized with prototype 1 using Elution I)</td>
<td></td>
</tr>
</tbody>
</table>

The buffer selected for the elution step in the experiments with prototype 2 was the elution buffer 1 (Buffer TE) because the best results in prototype 1 were achieved using it. Therefore, the three elution conditions using this buffer tested in prototype 1 were also tested in the prototype 2, namely Elution I (room temperature), III (room temperature and 5 minutes of incubation time) and IV (63°C and 5 minutes of incubation time). The results obtained with these tests after quantification using PicoGreen® are illustrated in Figure 4.17.

The results obtained by testing these three elution conditions did not show big differences on the amount of eluted DNA, resulting in similar efficiencies (1.6%, 1.3% and 1.6% for Elution I, III and IV, respectively) independently of incubation times or temperatures, which do not corroborate the results achieved with the prototype 1.

Since the elution efficiency did not vary with the tested conditions, it was considered the hypothesis of the problem being related with the efficiency of the binding step. This prototype is much smaller than the other one and, consequently, the fluids pass through the system more rapidly. This means that neither the initial DNA sample neither the introduced buffers have a residence time as long as in the prototype 1, which may lead to an inefficient binding of DNA to the membrane, being lost majorly in this step.
**Alternative Protocol III**

Since the binding of DNA to the silica membrane using chaotropic buffers is much stronger than using non-chaotropic buffers, the Alternative Protocol III was also tested in the prototype 2 in order to verify if the binding step and, consequently, the elution efficiency were improved in these conditions. The volumes described in the Alternative Protocol III were also reduced, being used volumes similar to the ones described for the modified Alternative Protocol IV in Table 4.13.

The only chaotropic binding buffer tested in the prototype 2 using the Alternative Protocol III was BB3 because, in the prototype 1, this was the chaotropic buffer with best extraction results using stdDNA solutions. Therefore, the three elution conditions described before were tested with this device using BB3 as binding buffer and the results are presented in Figure 4.18.

Although the residence time of the binding buffer was the same in both alternative protocols, more DNA was bound to the membrane using the chaotropic buffer however the elution efficiency was lower than in the previous protocol. These results show that the binding efficiency obtained using chaotropic buffers is much higher compared with the non-chaotropic buffer used in Alternative Protocol IV but the amount of DNA eluted when using chaotropic buffers for binding was, once again, lower than with non-chaotropic binding buffers, which corroborates the results obtained in the prototype 1. The elution efficiencies determined for each elution condition tested using alternative protocol III are summarized in Table 4.15.

![Graph](image)

**Figure 4.18 - Test of different elution conditions using Alternative Protocol III with prototype 2.**

<table>
<thead>
<tr>
<th>Elution Efficiency (%)</th>
<th>Elution I</th>
<th>Elution III</th>
<th>Elution IV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.75</td>
<td>0.83</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Table 4.15 – Elution Efficiencies obtained for each elution condition tested with Alternative Protocol III in prototype 2.
Although the Elution IV was slightly better, there were not big differences relatively to the other conditions. Once again, the low efficiencies obtained may be related with the residence time of the buffers, especially in the elution step because using chaotropic binding buffers makes the DNA elution more difficult to achieve.

4.3.3. Effect of Membrane Characteristics on DNA Yield

Two different types of commercial silica membranes were tested with the prototype 2 in order to analyze their influence in the extraction process. The membrane I was thinner but had smaller pores than membrane II, as described in the previous chapter. These two membranes were tested using both chaotropic and non-chaotropic buffers by applying the Alternative Protocols III and IV, respectively.

The Alternative Protocol III was performed in triplicate using BB3 and Elution III, which included 5 minutes of incubation at room temperature. The results obtained for both membranes after quantification with PicoGreen are illustrated in Figure 4.19.

![Figure 4.19 – Test of Membranes I and II with Alternative Protocol III using BB3 and Elution III in prototype 2.]

The Alternative Protocol IV was also performed in triplicate using Elution III. The obtained results are presented in Figure 4.20.

![Figure 4.20 - Test of Membranes I and II with Alternative Protocol IV using BB4 and Elution III in prototype 2.]

64
The extraction efficiencies obtained for both membranes in each protocol are summarized in Table 4.16.

<table>
<thead>
<tr>
<th>Protocols</th>
<th>DNA extraction efficiency (%)</th>
<th>Membrane I</th>
<th>Membrane II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternative III</td>
<td>0.83</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td>Alternative IV</td>
<td>1.3</td>
<td>0.33</td>
<td></td>
</tr>
</tbody>
</table>

The results of both tests were consistent, showing that the extraction with membrane I had a higher elution efficiency than with membrane II independently of using chaotropic or non-chaotropic binding buffers. However, the difference between the two membranes was not big using the Alternative Protocol III, suggesting that although the pores of membrane II are bigger its thickness compensates the pores size. On the other hand, using the Alternative Protocol IV, the DNA extraction yield was 4 times bigger with Membrane I than Membrane II, suggesting that the characteristics of the silica membrane have a bigger effect on DNA extraction efficiency when using non-chaotropic binding buffers.

Once again, the amounts of DNA eluted were very low, resulting in low extraction yields and suggesting that a lot of DNA was still bound to the membrane. Higher incubation times for binding and elution steps should be tested.
CHAPTER 5. CONCLUSION AND FURTHER WORK

5.1. Conclusion

The work described in this thesis was focused on the development and optimization of a DNA extraction protocol suitable for integration in a microfluidic setting based on microscale solid phase extraction (μSPE). The type of solid-phase selected for this work was a silica membrane because it is easier to integrate in a microfluidic device and it is widely used at bench scale, which allowed the comparison between the results obtained with the microdevices and with the commercial kits. The comparison and optimization of the tested protocols was achieved by using a stdDNA solution to determine the extraction yields. The development of this work was intended for food analysis applications therefore, after optimizing the protocols using stdDNA solutions, sesame seeds were also tested as an example of a challenging food sample.

The initial protocol, developed based on the DNA extraction procedure used in commercial kits, included a binding step using chaotropic buffers, a washing step with ethanol, a drying step to remove the remaining washing buffer and, finally, an elution step. This protocol was applied in both prototypes and it was demonstrated that the presence of air bubbles inside these systems is a major disadvantage for a DNA extraction process in microfluidics, introducing variability of results and limiting the integration of this process with further DNA analysis techniques.

Therefore, a set of DNA extraction protocols without drying step was developed and tested using the prototype 1 in order to avoid the introduction of air into the system. From the set of alternative protocols tested the alternatives III and IV achieved the highest elution yields. The alternative protocol III included chaotropic binding buffers and two washing steps before the elution phase. The first washing step used ethanol in order to remove the unbound components and the excess of chaotropic salts present in the system, while the second wash used a non-chaotropic buffer to remove the ethanol remaining from the previous step. On the other hand the alternative protocol IV used the non-chaotropic buffer as binding and also as washing buffer. These two approaches allowed a comparison of the effect of chaotropic and non-chaotropic buffers on the DNA extraction yield. As a conclusion, the chaotropic buffers demonstrated higher binding efficiency than non-chaotropic buffers, as expected. However, a stronger binding of DNA to the membrane resulted in a more difficult elution. This relation was demonstrated by the alternative IV because, although more DNA was lost during the binding step, the amount of DNA collected in the elution step was similar to the amount eluted using the alternative protocol III with chaotropic binding buffers.

These two approaches were then optimized by reducing the volume of washing buffer in order to minimize the amount of DNA lost during this step (protocols III and IV) and by doing a pre-treatment of the membrane to improve the binding efficiency (protocol IV). These modifications proved to be able to enhance the DNA extraction yield using the prototype 1 and, as a result, the efficiencies increased from 4.4 to 8.9 % and from 14
to 44% in the alternative protocols III and IV, respectively. In addition to the optimization of binding and washing steps, a set of elution conditions was tested and the results showed that incubation time and temperature affect the elution yield. It was demonstrated that an elution at 63°C and with 5 minutes of incubation time can improve the efficiency of the extraction, which increased the yield of protocol IV to 45.8 %. Both elution buffers tested in these experiments, namely Buffer TE and DI water, demonstrated good elution results.

Sesame seeds were used as a food sample to challenge the prototype 1 by applying the initial protocol and the optimized one. The amount of DNA eluted using the initial protocol was higher than with the alternative IV after optimization, which did not corroborate the results obtained using stdDNA solutions. However high variability was observed in the elution step of the initial protocol, probably related with the presence of air bubbles during this phase, resulting in poor reproducibility. Moreover, sesame seeds and stdDNA solutions are two very different matrices, which may be the reason for the differences observed in their extraction results. Since sesame seeds are more complex, there are much more components present in the lysate besides DNA. By comparison of the results obtained with the prototype 1 and with the commercial kits, the extraction of DNA from sesame seeds was better using the last ones regarding both purity and ratio of DNA mass (ng) to sesame seeds mass (mg). These results proved that the type of matrix has a big influence on the extraction process and showed that more tests are required in order to improve the DNA extraction from sesame seeds using these microfluidic systems.

The prototype 2 was fabricated and tested aiming to reduce the extraction time as well as the volumes required for the process. The extraction time was reduced by half in the prototype 2 when compared to the prototype 1. The first tests performed using the prototype 2 suggested that the collected volume in each step should be decreased to avoid diluting the samples. It also indicated that the volume of DNA sample initially introduced should be reduced because, with a smaller membrane, the surface area for DNA binding is lower than in the bigger device (prototype 1), which contributes for low efficiency results. However, with further tests, it was demonstrated that other features must be taken into account, namely the residence time of samples and buffers. Considering the size of this device, transferring the protocol defined for the prototype 1 to the prototype 2 is not enough to achieve the expected results. In a smaller system like prototype 2, the fluid passes through the membrane more rapidly because its chamber volume is lower, resulting in an inefficient binding and elution steps, which suggests that higher incubation times must be included in these steps or lower flow rates must be selected.

The two types of silica membrane tested in the prototype 2 had different pore size and thickness and the obtained results demonstrated that the extraction using membrane I (smaller pores and lower thickness) was more efficient than membrane II (bigger pores and higher thickness) for both alternatives III and IV. The difference between the results using alternative protocol III was not very big, suggesting that a higher thickness may compensate a higher pore size. However, the DNA extraction efficiency obtained using the alternative protocol IV with Membrane I was approximately 4 times bigger than with Membrane II, suggesting that the
characteristics of the silica membrane have a bigger effect on DNA extraction yield when using non-chaotropic binding buffers.

All the quantification measurements were performed using the PicoGreen® method, which is very sensitive and specific for dsDNA. However this method required calibration curves with DNA standards for each quantification experiment and the slopes of these standard curves varied from day to day even using the same calibration standards every time. This variation may lead to slightly different results for the same sample depending on the obtained curve. In addition, during the quantification experiments it was found that the presence of chaotropic salts in the samples resulted in signal quenching. Therefore, it is important to consider that the quantification results of samples with chaotropic salts in solution may be underestimated. In spite of this, the quantification results of the elution samples and, consequently, the elution yields should not be affected by this limitation because the elution buffers used do not contribute for this effect, since chaotropic salts are not present.

Given these points, in order to develop microfluidic systems integrating extraction and amplification procedures, these two techniques must be optimized separately for a better understanding of the features that affect them when performed at microscale. This work was based on this modular approach and aimed the optimization of DNA extraction protocols, regarding the integration of further steps of DNA analysis.

In conclusion, the work presented in this thesis described the development of an optimized protocol suitable for DNA extraction based on the microscale solid phase extraction technique using a washable and reusable microfluidic system. This protocol involved a non-chaotropic binding buffer, an ethanol free washing step and a short incubation with buffer TE for DNA elution, achieving the highest extraction yield and the best feasibility for a microfluidic setting. More tests are required to improve the purity of DNA and the efficiency of the process when using food samples but this microdevice proved to be suitable for this purpose.

5.2. Further Work

There are several aspects that can be further tested in order to improve the work described in this project. Some of those are proposed below:

- More tests using sesame seeds and other complex matrices are required in order to improve the purity of the extracted DNA. For instance, the DNA purity obtained with the optimized protocol IV in the prototype 1 may be enhanced by increasing the volume of washing buffer to better remove the unbound contaminants.
- In the prototype 2, protocols using longer incubation periods in the binding and elution steps should be tested to analyze their effect on the extraction efficiency. These longer incubation times would allow the buffers to be in contact with the membrane for longer periods under specific conditions.
- Silica membranes with different characteristics should also be tested (e.g.: different pore size).
In order to optimize the time required for DNA extraction, a set of different flow rates should be tested for each step of extraction. It is important to find a balance between the rapidness of the process and the ability to not detach the DNA from the membrane as well as to give enough incubation time for contact between the buffers and the membrane.

Tests using more complex food matrices as samples should be performed to challenge the microfluidic system and evaluate its applicability. For example, mixtures of regular flour and sesame seeds could be used to increase the complexity of the food samples for DNA extraction.
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