

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

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Abstract

The present work describes the development and optimization of a DNA extraction protocol based on microscale solid phase extraction (μ SPE) for food analysis applications. Two washable and reusable microfluidic systems, containing commercial disposable silica membranes, were used to optimize each step of DNA extraction: binding, washing and elution. A set of protocols that could be integrated onto micro Total Analysis Systems (μ TAS) was tested. These protocols were compared regarding their extraction efficiency, DNA purity and suitability for integration in a microfluidic setting. A DNA extraction procedure at bench scale was also performed, for comparison with the results at microscale, by using a protocol adapted from two commercial kits, namely NucleoSpin® Food and NucleoSpin® Plant II. One of the protocols tested at microscale, 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.

Introduction

In the past few decades, several safety crises in the food sector, such as the bovine spongiform encephalopathy (BSE), also known as “mad cow” disease (Nathanson, et al., 1997), the dioxin contaminated feed (Bernard, et al., 2002) and, more recently, the *E. coli* 0104:H4 outbreak in 2011, highlighted the need for a more rigorous food safety and quality control system, making evident the importance of being able to rapidly identify and isolate unsafe food products to prevent them from continuing to reach the consumers market. As a result, a new strategy was developed based on assurance of food traceability from farm to fork, 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). With the world-wide integration of food supply chains, this ability is especially important because it allows the identification of the origin of food crises, giving more confidence to the consumers and, at the same time, allowing food control authorities to take all the required preventive and corrective measures.

Nowadays, consumers are more aware of the importance of a good food security and quality control system and they want to be informed about the food they buy, however they have to rely on product labeling and advertising to get information about the nature of the products.

Food authenticity is the verification of conformity between the product content and the label information provided, helping to prevent food fraud, which has been rising in the last years and has very important economic and social consequences. A recent example of a food fraud incident was the horsemeat scandal in 2013, where frozen beef-burgers and beef-labeled ready meals contained up to 100% horsemeat, affecting several countries in Europe (European Commission, 2013). Mislabeled allergenic components resulting from undeclared ingredient substitution are also very common frauds and may have serious consequences.

Food authenticity issue, in particular in foods of added value, such as olive oil, has become one of the major concerns of the food industries. Olive oil is one of the products most at risk of food fraud therefore food authenticity and traceability are especially important for this market (Committee on the Environment, Public Health and Food Safety, 2013).

Moreover, the determination of the origin of a food product like olive oil and its production procedures is a guarantee of the product healthiness and safety (Busconi, et al., 2003).

The interest for DNA-based analysis applied to food authentication and detection of allergens and genetically modified organisms (GMOs) has increased significantly (Prado, et al., 2015), mostly due to the high stability and durability of DNA when compared with proteins. Moreover, DNA is species-specific, allowing the verification of species, and can be found in every cell of plants and animals which are the major constituents of food products (Prado, et al., 2007). The main steps for DNA-based analysis are extraction, amplification and quantification/detection of DNA. Amplification, which is usually achieved by polymerase chain reaction (PCR) techniques, is extremely dependent on the extraction procedure, which should be able to recover the nucleic acids and, at the same time, remove compounds that may inhibit amplification (Demeke & Jenkins, 2010).

Food products are complex samples composed by several ingredients with different properties, some of which may inhibit the DNA amplification, such as polysaccharides and humic acids. In addition, the processing procedures used in food production may affect the DNA quality, limiting the amount of DNA that can be extracted and amplified (Gryson, 2010).

Micro and nanotechnologies have been contributing to revolutionize many industry sectors, including food industry, by integrating one or more laboratory procedures in a single microdevice, using the lab-on-a-chip approach. Microfluidic systems are suitable for this purpose since they can be used for sampling, monitoring, control and transport as well as for mixing, reaction, incubation, and analysis of small volumes of fluids (Abgrall & Gué, 2007). Miniaturized DNA analysis systems need smaller sample and reagent volumes, improving the performance of the system by being faster and more sensitive. Moreover, they are more suitable for automation, decreasing the risk of contamination during the analysis process (Price, et al., 2009). Another advantage of these systems is their portability, providing *in situ* analysis.

DNA extraction is a critical step for any miniaturized DNA analysis system. The choice of the most adequate extraction method must be done according to the type of nucleic acid to be isolated, the type of sample to be analyzed and the purpose of its isolation, regarding downstream amplification and

detection processes. Solid-phase extraction (SPE) is an extraction method quite used in analytical chemistry, by which the compounds of interest present in a liquid mixture are separated from the other compounds by being retained on a stationary phase due to their higher affinity (Poole, 2003).

In this study, two washable and reusable microfluidic systems with an embedded silica membrane were tested using a set of different DNA extraction protocols, employing chaotropic and non-chaotropic buffers, based on microscale solid-phase extraction (μ SPE) for food analysis applications. The main steps of this type of extraction are the binding, washing and elution of DNA. The protocols were optimized using standard DNA (stdDNA) solutions and then tested with sesame seeds as an example of challenging plant/food sample. A DNA extraction procedure at bench scale was also performed by using a protocol adapted from two commercial kits, namely NucleoSpin® Food and NucleoSpin® Plant II from Macherey-Nagel. The results obtained at both bench and micro scales were then compared. The protocols developed and optimized with the microfluidic systems were also compared, concerning extraction efficiency, DNA purity and suitability for integration in a microfluidic setting.

Materials and Experimental Methods

BIOLOGICAL SAMPLES: The standard DNA used in this study was a low molecular weight DNA from salmon sperm (Sigma-Aldrich). The concentration of the stdDNA solution tested was 50ng/ μ l, being used for the optimization of the protocols. Sesame seeds were also tested in order to challenge the systems with a food sample.

COMMERCIAL KITS: A combination of the protocols of the NucleoSpin® Food and NucleoSpin® Plant II kits was used to perform the DNA extraction from sesame seeds. *Sesame seeds:* 200 mg of sample were homogenized and its lysis was performed by incubation with lysis buffer at 65°C for 3h with interval mixing, by following the NucleoSpin® Food protocol. The lysate was then filtered according with the NucleoSpin® Plant II protocol and the clear flowthrough was used for the binding and washing steps as described in NucleoSpin® Food protocol. The elution was performed by adding 50 μ L of elution buffer pre-heated to 65°C, waiting 5 minutes at room temperature and then centrifuging for 1 min at

11000xg. This elution step was repeated resulting in a final volume of 100 μ L of eluted DNA solution, according with the NucleoSpin[®] Plant II protocol. *StdDNA solution*: protocol was similar to the one used for the sesame seeds but without the lysis and filtration steps.

MICROFLUIDIC DEVICES: two washable and reusable microfluidic devices were tested using a set of different protocols based on μ SPE with disposable silica membranes.

- **Design and Experimental Set up:** Both microfluidic systems were designed and fabricated in poly (methyl methacrylate) (PMMA) in collaboration with the MEMS research group at INL, having one inlet channel and one outlet channel. The prototypes were connected to a syringe pump with a constant flow rate for each step of DNA extraction. The fluid introduced in the inlet channel passes through the silica membrane and is released through the outlet channel. In order to avoid fluid leakages, one o-ring was placed under the membrane and another one was placed over it, while a seal was used to cover the region around the membrane and both channels. The seals and o-rings were produced in polydimethylsiloxane (PDMS) while the molds for their production were also fabricated on PMMA. The big difference between the two devices is their dimensions. 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 μ L. On the other hand, 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 μ L. The design of these systems is illustrated in Figure 1 (A).

- **Assembly and Flow Rate Test:** The PDMS seals and o-rings were produced with variable thicknesses (0.5, 0.75 and 1 mm) in order to test the most adequate for our experiment. The liquid PDMS was poured into the molds, placed in a plastic vacuum desiccator to remove the air bubbles and then cured in the oven at 65°C for 1h. The best assembly option was then tested within a range of flow rates (50-500 μ L/min) in order to verify if the prototypes could work under these conditions without fluid leakage. These tests were performed using dye solutions (Figure 1 (B)).

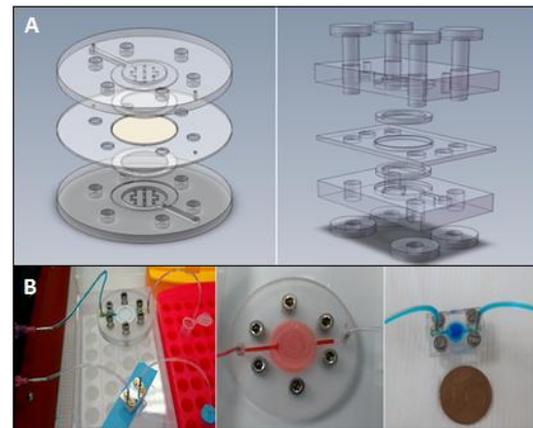


Figure 1 – (A) 3D Design and assembly structure of prototypes 1 and 2. **(B)** Assembly and flow rate tests using dye solutions.

- **Buffers Composition:** Before the extraction experiments, a pre-conditioning step was performed by passing a 0.1% (v/v) Tween-20 solution followed by DI water (Milli-Q). For the DNA extraction tests, three chaotropic binding buffers (BB1, BB2 and BB3) and one non-chaotropic binding buffer (BB4) were prepared. Guanidine thiocyanate (GuSCN) is a chaotropic salt that provides high binding affinity for nucleic acids on silica therefore it was selected for this study. *Chaotropic Binding Buffers:* BB1 (6M GuSCN, Buffer TE (10mM Tris-HCl, 1mM EDTA), 50% EtOH; pH 6.4), BB2 (6M GuSCN, Buffer TE; pH 6.4) and BB3 (6M GuSCN, 20mM EDTA pH 8, 10mM Tris-HCl pH 6.4, 4% TritonX-100; pH 7.3). *Non-Chaotropic Binding Buffer:* BB4 (0.25M Glycine, 400 mM KCl; pH 5). *Washing Buffer:* EtOH 85% (v/v). *Elution Buffers:* E1 (Buffer TE; pH 8) and E2 (DI water (Milli-Q)). Alternative protocols were developed using some of the described binding buffers also as washing buffers.

- **Sample Preparation:** *stdDNA solutions* do not need any preparation before starting the extraction protocol, being added to the binding buffer that is going to be tested in a ratio 1:1 and then introduced in the microfluidic device. *Sesame seeds* require a previous preparation to lyse the cells and release the DNA. The lysis protocol depends on the binding buffer composition. The BB1 and BB3 can be used as lysis buffers since they include ethanol and Triton X-100 in their composition, which are able to induce the cell disruption process (Ghosal & Srivastava, 2009). In this case, 1500 mg of previously grinded sesame seeds were mixed with 4 ml of binding buffer and incubated 1h30 at 65°C with agitation. Then the lysate was centrifuged at 4000 rpm for 10 minutes and the clear liquid in the middle was collected and introduced in the system while the pellet including

the solid remains from the cell walls, together with the upper portion with low density components were discarded. For tests with BB2 and BB4, the lysis was performed with the commercial kits by following the protocol until the filtration step and, then, the filtered lysate was added to the corresponding binding buffer in a ratio 1:1. This protocol was 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.

- **Initial Protocol:** The initial protocol was based on the extraction procedure performed in the commercial kits and it was tested in both microfluidic systems after the preparation of the samples. This protocol is composed by binding, washing, drying and elution steps and the flow rates applied in each step were 10, 20, 200 and 10 μ l/min, respectively. The drying was achieved by passing air through the system to remove the ethanol remaining from the washing process. In the prototype 1, the volumes passed through the device in the binding, washing and elution steps were 1000, 2000 and 2000 μ l, respectively, while in the prototype 2 were 1000 μ l in each phase. Samples were collected in each step for later quantification. The average volume of each sample collected in the prototype 1 was 500 μ l and, in the prototype 2, was 250 μ l.

- **Alternative Binding and Washing Protocols:** Four alternative protocols were developed in order to remove the drying step needed in the initial one, either by the substitution of ethanol as a washing buffer or by the addition of a second wash to remove the ethanol remaining from the first one. These alternatives were tested with the prototype 1 using the buffer E1 for the elution step. *Alternative I:* chaotropic buffer (BB2) was used not only in the binding but also in the washing step, replacing the wash with ethanol. *Alternative II:* chaotropic buffer (BB2) was used in the binding step while the washing phase was performed in two steps, by first washing with ethanol and then with the chaotropic buffer used for binding. *Alternative III:* chaotropic buffers (BB1, BB2 and BB3) were used in the binding step, then, a first washing step was performed by using ethanol while the second wash used the non-chaotropic buffer (BB4). *Alternative IV:* the non-chaotropic buffer (BB4) was used for binding and washing steps. The flow rates applied in the binding, washing and elution steps for testing these alternative protocols were the same used in the initial

one. The alternative protocols described were then optimized, also in prototype 1, by reducing the volume of the buffers used in the washing steps (Alternatives III and IV) and by doing a pre-treatment of the silica membrane, which consisted in passing the BB4 buffer through the system before starting the binding step (Alternative IV). In the prototype 2, the Alternatives III and IV, previously optimized with the other prototype, were tested. In order to optimize the obtained results, the volumes collected in each sample were reduced to 100 μ l, instead of 250 μ l as in the initial protocol, to avoid collecting too diluted samples. The volumes initially collected were higher than the volume capacity of the device and, 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. Therefore, the volumes of buffers introduced in the prototype 2 in each step were also reduced from 1000 μ l to 500 μ l.

- **Alternative Elution Protocols:** After optimizing the binding and washing phases of DNA extraction, four elution conditions were tested in order to improve this step in the prototype 1. *Elution I:* the buffer E1 was used as elution buffer and this step was executed at room temperature. *Elution II:* the buffer E2 was used as elution buffer and the elution step was also performed at room temperature. *Elution III:* the buffer E1 was used for elution with a 5 minute incubation time at room temperature. *Elution IV:* this step was performed with the same conditions as elution III but with a temperature of approximately 63 $^{\circ}$ C. For the prototype 2, just the conditions described by Elution I, III and IV were tested.

- **Test with different silica membranes:** All the tests performed in the prototype 1 were done using the same type of silica membrane (Membrane I) while, in the prototype 2, two commercial silica membranes (GE Healthcare Life Sciences) with different characteristics were tested. *Membrane I (GF/A):* 260 μ m of thickness and 1.6 μ m of particle retention in liquid. *Membrane II (GF/D):* 675 μ m of thickness and 2.7 μ m of particle retention in liquid. The Membrane I (GF/A) was cut to fit in the prototype 2.

QUANTIFICATION AND PURITY OF DNA: The DNA extracted using the commercial kits, and the samples collected in each step of the DNA extraction experiments using the microfluidic systems, were quantified with the

Quant-iT™ PicoGreen® dsDNA assay kit (Invitrogen). When PicoGreen® binds to the dsDNA in solution a very strong increase in fluorescence is detected. In order to correlate the emitted fluorescence with the dsDNA concentration in the samples, a standard curve was obtained by measuring serially diluted dsDNA standards with the following final concentrations: 0, 1, 5, 10, 25, 100, 500 and 1000ng/ml. The quantification experiments used an excitation maximum of 470 nm and an emission maximum of 525 nm. The initial stdDNA solution (50ng/μl) was also quantified using the Quant-iT™ PicoGreen® dsDNA assay kit to confirm the concentration. In order to have a value inside the range of concentrations used to obtain the calibration curve (0-1000ng/ml) this solution was diluted by a factor of 100. As a result, the diluted stdDNA solution (500ng/ml) was measured using the PicoGreen® reagent and the average concentration obtained was 10.75ng/ml. After correction with the dilution factor, the value corresponding to the initial concentration was 1075ng/ml, being underestimated and showing that the reagent was not reacting as well as expected. Since this technique was 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.

The stdDNA solution was also quantified using a spectrophotometer (NanoVue™), and the expected concentration was obtained, proving that the solution preparation was done correctly.

Then, the ratio of absorbances at 260nm and 280nm (A260/A280) was determined by using the Thermo Scientific NanoDrop 2000 spectrophotometer in order to evaluate the purity of the DNA extracted from sesame seeds. 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 necessarily mean that the DNA is not suitable for downstream applications.

Results and Discussion

DNA extraction protocols were tested in triplicate and optimized in terms of buffer composition,

applicability for automation on a microfluidic device and collected volume for higher DNA yields.

ASSEMBLY AND FLOW RATE TEST: The best assembly option in order to avoid leakages was the use of both o-rings and seal with 1mm of thickness. The flow rate test performed in both prototypes using this assembly option demonstrated that the range of flow rates applied was supported by the systems, without observed leakage.

COMMERCIAL KITS FOR DNA EXTRACTION: DNA extraction from the stdDNA solution using the commercial kits was achieved with a DNA yield of 10.5%. As an example of challenging food sample, sesame seeds were also used for DNA extraction resulting in an average of 0.39ng extracted DNA/mg sesame seeds with an average purity ratio (A260/A280) of 1.89.

MICROFLUIDIC SYSTEM: PROTOTYPE 1

In the prototype 1, the protocols were firstly optimized with the stdDNA solution and then tested using sesame seeds in order to challenge the system with a food sample.

- **stdDNA Solution:** the initial protocol was tested with this sample for the optimization of binding step using different chaotropic buffers (BB1, BB2 and BB3) and, then, the washing step was optimized using a set of alternative protocols, including the use of a non-chaotropic buffer (BB4). Finally, the optimization of elution step was performed by testing a set of different conditions.

Optimization of Binding Step

The Initial Protocol, previously described, was tested with the chaotropic binding buffers (BB1, BB2 and BB3) in order to select the one with the best binding efficiency. The results demonstrated that, although some DNA was lost in the binding step, low amounts were released in washing and drying step and the phase in which most DNA was collected was the elution step as expected (Figure 2 (A)). BB3 had the best binding results but BB1 was the buffer that provided the best extraction efficiency (10.3%). However, high standard deviations were observed with this protocol due to the variability observed between the triplicates, especially in the drying and elution steps, which might be related with the presence of air bubbles in the system. The drying step is required in this protocol to remove the ethanol remaining from the wash, which is a PCR inhibitor, however the presence of air bubbles not only affects

the extraction efficiency, since it reduces the membrane surface area contacting with the elution buffer, but also is a limitation for the integration of further DNA analysis steps in a microfluidic setting.

Optimization of Washing Step

A set of alternatives, previously described, were tested to optimize the washing step and, consequently, remove the drying step needed in the initial protocol. The results of Alternatives I and II, tested using BB2, demonstrated that although the protocol was easier to perform due to the removal of the drying step, no elution was achieved. These results may be explained by the fact that the binding buffer used was a strong chaotropic buffer and, since it was also used in the washing step, when the elution occurred there was still too much salt present in the system and the conditions for DNA elution were not favorable, consequently, the DNA remained bound to the membrane instead of being collected during the elution. The first tests with Alternative III used BB2 as binding buffer and the second wash was performed with the non-chaotropic buffer (BB4), as a result, some DNA was collected during the elution step and a DNA yield of 4.4% was achieved, which supports the explanation for the results obtained in the previous alternatives. The binding of DNA to the silica membrane is not as strong in non-chaotropic conditions as it is with chaotropic buffers, thus BB4 was able to remove the ethanol without completely limiting the elution. However, too much DNA was lost during the second wash (Figure 2 (B)). Finally, Alternative IV was tested using the non-chaotropic buffer (BB4) for both binding and washing steps, resulting in a DNA yield of 14% (Figure 2 (D)). Although the adsorption of DNA to the silica membrane was stronger using the chaotropic buffers, the amount of DNA eluted with this alternative was similar to one using the initial protocol. However, too much DNA was lost during the binding and washing steps, leaving room for further optimization.

Combined Optimization of Binding and Washing Steps

Although Alternatives III and IV showed the best results to remove the drying step, these protocols could still be improved, especially regarding the binding and washing phases. This optimization was obtained by reducing the volume of washing buffer

and/or adding the membrane pre-treatment step described before. Optimization of Alternative III was achieved by reducing the volume of the first wash (EtOH) from 2000 to 1000 μ l and the second wash (BB4) from 1500 to 1000 μ l, resulting in a DNA yield of 6.6% (Figure 2 (B)). This test was performed using BB2 for the binding step but the other chaotropic buffers were also tested and the results demonstrated that BB3 was the best binding buffer to apply in this protocol (Figure 2 (C)), improving the extraction efficiency from 4.4% to 8.9% when compared with the non-optimized protocol. Optimization of Alternative IV was firstly performed by reducing the volume of washing buffer from 2000 to 1000 μ l, resulting in a DNA extraction yield of 35%. The amount of DNA lost in the binding step was too high and, therefore, the membrane pre-treatment step was also added to the protocol, improving the DNA extraction efficiency from 14% to 44% when compared with the non-optimized protocol (Figure 2 (D)).

Optimization of Elution Step

After optimization of binding and washing steps, the elution was also optimized by testing the set of conditions previously described (Figure 2 (E)). The tests with Elution I and Elution II were performed in the same conditions, at room temperature and without incubation, but different elution buffers were used. Although buffer E1 (TE) showed a better efficiency than E2 (DI water), both elution buffers demonstrated good results (44% and 32.6%, respectively). The best efficiency was achieved by the conditions used in Elution IV (45.8%), which included 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). However, the efficiency determined for Elution III (with 5 minutes incubation at room temperature) seems to be lower than the one obtained for Elution I (without incubation). This result does not corroborate the previous conclusion, which 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.

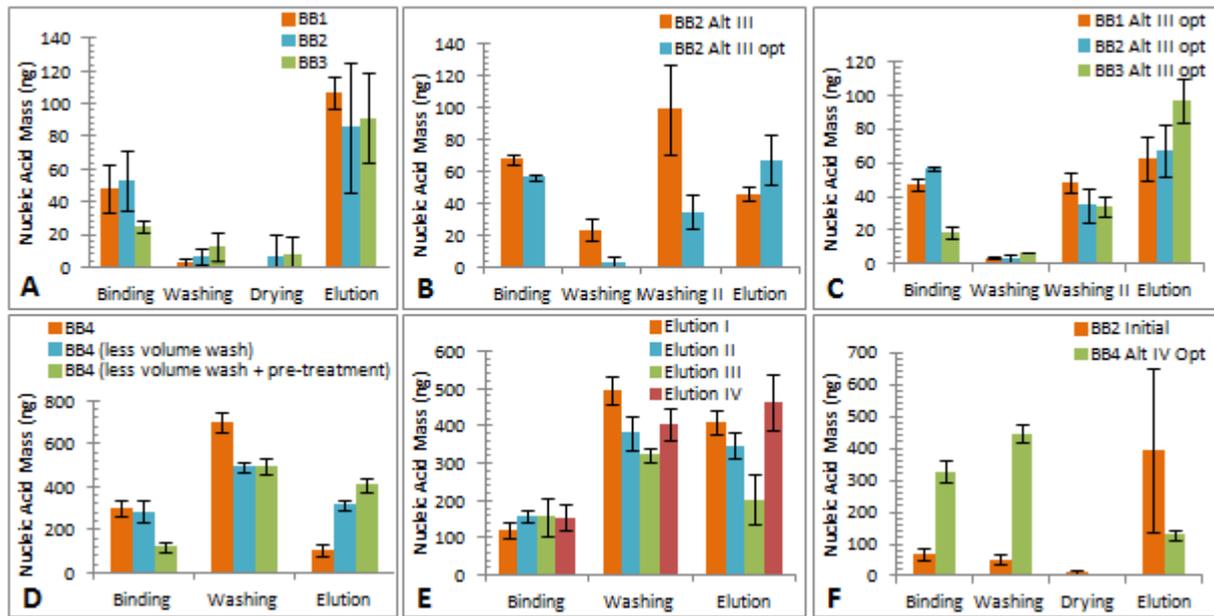


Figure 2 - Tests with Prototype 1: (A) Test of Initial Protocol with stdDNA using chaotropic binding buffers (Yields: BB1=10.3%, BB2=8.9%, BB3=9.4%); (B) Optimization of washing steps with stdDNA in alternative protocol III through the reduction of washing volume (Yields: BB2 AltIII=4.4%, BB2 AltIII opt=6.6%); (C) Test with chaotropic binding buffers using alternative protocol III after optimization with stdDNA (Yields: BB1 AltIII opt=5.8%, BB2 AltIII opt =6.6%, BB3 AltIII opt =8.9%); (D) Optimization of washing step through the reduction of washing volume and optimization of binding step with pre-treatment of the membrane in Alternative Protocol IV (Yields: BB4=14%, BB4 less vol. wash=35%, BB4 less vol. wash+pre-treat.=44%); (E) Optimization of elution step with stdDNA using Alternative Protocol IV after optimization of binding and washing steps (Yields: Elution I=44%, Elution II=32.6%, Elution III=20.2%, Elution IV=45.8%); (F) DNA extraction from sesame seeds using the Initial Protocol with chaotropic binding buffer BB2 and using the Alternative Protocol IV after optimization (Yields: BB2 Initial=0.71 ng DNA/mg sesame seeds, BB4 AltIV opt=0.23 ng DNA/mg sesame seeds; Purity A260/A280: BB2 Initial=1.05, BB4 AltIV opt=1.12).

- **Sesame Seeds:** The initial protocol was tested using sesame seeds as sample and, then, the Alternative IV, after optimization, was also performed for comparison. With the Initial Protocol, the best results were obtained using BB2, since more DNA was collected in the elution step and less was lost in the previous phases. In the tests using BB1 and BB3, the formation of a precipitate during the binding step was noticed. This was not observed with BB2, what could be explained by the differences in the preparation of the samples, such as the addition of proteinase K in the lysis used for the test with BB2, resulting in the degradation of proteins. Chaotropic agents 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), promoting their precipitation. 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, therefore, the results from the tests with BB1 and BB3 were inconclusive. The optimized Alternative IV was also used to extract DNA from sesame seeds. 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 (Figure 2 (F)). The amount of DNA eluted using the initial protocol (0.71 ng DNA/mg sesame seeds with A260/A280=1.05) was higher than with the optimized Alternative IV (0.23 ng DNA/mg sesame seeds with A260/A280=1.12). These results do not corroborate the ones obtained using the stdDNA solution as a sample, however, it is important to consider that this solution and sesame seeds are two very distinct matrices. In addition, high variability was observed in the elution step using the Initial Protocol, which might be related with the presence of air bubbles during the elution, being introduced with the drying step. The Alternative IV did not need drying because an ethanol-free wash was used therefore the variability between the triplicates was much lower and, consequently, the reproducibility of results was enhanced. Moreover, this alternative is more suitable for a microfluidic setting and for integration of further steps of DNA analysis than the initial protocol.

MICROFLUIDIC SYSTEM: PROTOTYPE 2

The prototype 2 was developed as a miniaturization of prototype 1, bringing several

advantages, namely the reduction of sample and buffer volumes required and the ability to perform the DNA extraction more rapidly. Although the extraction time of prototype 1 was reduced by optimization, it still required a long time (approximately 6 hours), which could be reduced even more by using a smaller device. In the prototype 2, only stdDNA solutions were tested.

- **stdDNA Solution:** 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 and optimized according to the obtained results. Finally, two types of silica membranes were used to analyze their influence on the DNA extraction yield.

Test of Initial Protocol

Using the initial protocol, the chaotropic buffer BB2 achieved the best results in every step of the extraction and, consequently, the best elution yield (1.6 %) (Figure 3(A)). However, the reproducibility of results was not very good due to the presence of air bubbles, which had a bigger effect on the prototype 2 than on the big one, because in the smaller device the bubbles covered the entire membrane surface while, in the bigger one (prototype 1), the membrane was not completely covered. Thus, the surface interaction between the solutions and the membrane was considerably reduced.

Test and Optimization of Alternative IV

The Alternative IV was the first alternative protocol tested in the prototype 2 because it achieved the best results in the prototype 1. The first tests were performed applying this alternative protocol with the optimized binding and washing steps and collecting, in each step, samples with volumes of approximately 250 μl , passing a total of 1000 μl of sample or buffer volume in each step of extraction. However, the amount of DNA collected during elution was very low, almost undetectable (DNA yield: 0.47%) (Figure 3(B)). Since the DNA is majorly eluted in the first volume fractions of elution step, the following collected volumes may contribute to dilute the sample. For that reason, the volume of the collected samples was reduced to 100 μl and the volumes of introduced sample and buffers were reduced to 500 μl . With these alterations, the amount of eluted DNA, compared with the amount of DNA

introduced with the initial sample, was improved however the extraction yields were still too low (1.6%) (Figure 3(B)). Thus, the optimization of elution step was performed by testing three conditions: Elution I, Elution III and Elution IV (Figure 3(C)). The results obtained by testing these three conditions did not show big differences on the amount of eluted DNA, resulting in similar efficiencies independently of incubation times or temperatures, which do not corroborate the results achieved with the prototype 1. As a result, it was considered the hypothesis of the problem being related with the efficiency of the binding step because, since this prototype is much smaller than the other one, the fluids pass through the system more rapidly. Consequently, 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.

Test and Optimization of Alternative III

Since the binding of DNA to the silica membrane using chaotropic buffers is much stronger than using non-chaotropic buffers, the Alternative III was also tested in the prototype 2 in order to verify if the binding step and the elution efficiency were improved in these conditions. For this test, the volume of the collected samples was also 100 μl and the volumes of introduced sample and buffers were also 500 μl . The chaotropic buffer used was BB3 because it achieved the best binding results in the prototype 1. Therefore, the same elution conditions tested with Alternative IV were also tested with Alternative III (Figure 3(D)), resulting in lower amounts of DNA eluted. 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, which corroborates the results observed in the prototype 1. The Elution IV achieved the best extraction efficiency (1.1%), but there were not big differences relatively to the other conditions and the obtained DNA yields were very low. Further experiments using the prototype 2 should be performed in the future applying larger incubation times for both binding and elution steps.

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 yield. Membrane I

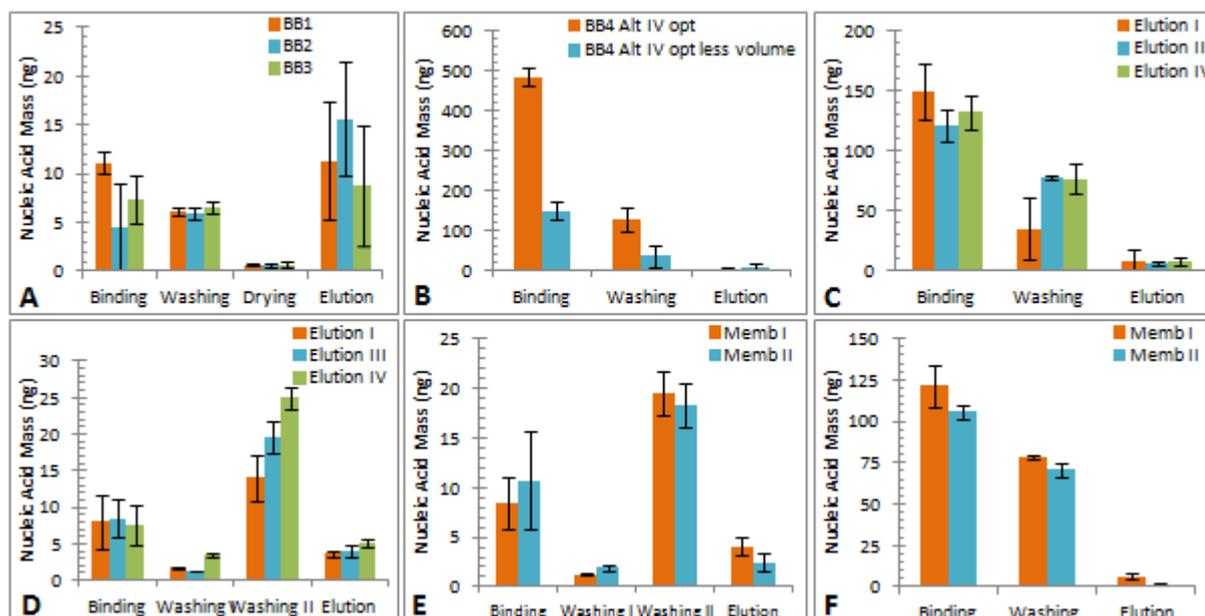


Figure 3 - Tests with Prototype 2: (A) Test of Initial Protocol using chaotropic binding buffers (Yields: BB1=1.2%, BB2=1.6%, BB3=0.91%); (B) Test of Alternative Protocol IV using less volume in binding, washing and elution steps (Yields: BB4 AltIV opt=0.47%, BB4 AltIV opt less vol.=1.6%); (C) Test of different elution conditions using Alternative Protocol IV (Yields: Elution I=1.6%, Elution III=1.3%, Elution IV=1.6%); (D) Test of different elution conditions using Alternative Protocol III (Yields: Elution I=0.75%, Elution III=0.83%, Elution IV=1.1%); (E) Test of Membranes I and II with Alternative Protocol III using BB3 and Elution III (Yields: Memb I=0.83%, Memb II=0.51%); (F) Test of Membranes I and II with Alternative Protocol IV using BB4 and Elution III (Yields: Memb I=1.3%, Memb II=0.33%).

was thinner but had smaller pores than Membrane II. These two membranes were tested using Alternative III (Figure 3(E)) and Alternative IV (Figure 3 (F)). The results of both tests were consistent, showing that the extraction with membrane I had a higher elution yield than with membrane II, independently of using chaotropic or non-chaotropic binding buffers. However, the difference between the two membranes using the alternative III was not big while the DNA extraction yield obtained using the alternative IV with Membrane I was approximately 4 times bigger than with Membrane II.

Conclusions

The tests performed in both microfluidic devices using the Initial Protocol demonstrated that the presence of air bubbles inside these systems due to the drying step 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.

From the set of alternative protocols tested, the alternatives III and IV achieved the highest elution yields. The results of these two approaches demonstrated that chaotropic buffers have higher binding efficiency than non-chaotropic buffers, as expected. However, a stronger binding of DNA to the

silica membrane resulted in a more difficult elution in both microfluidic systems. The optimization of the binding and washing steps in these two alternative protocols, by reducing the volume of washing buffer in order to minimize the amount of DNA lost during this step (Alternatives III and IV) and by doing a pre-treatment of the membrane (Alternative IV) as well as testing different binding buffers (Alternative III) to improve the binding efficiency, proved to be able to enhance the DNA extraction yields in the prototype 1. The efficiencies increased from 4.4 % to 8.9 % and from 14 % to 44 % in the alternative protocols III and IV, respectively. Moreover, the optimization of the elution step improved the yield of the Alternative IV to 45.8%, by performing an elution at 63°C and with 5 minutes of incubation time using TE buffer.

The DNA extraction from sesame seeds was performed in the prototype 1 using the Initial Protocol and the Alternative IV after optimization, however, the results did not corroborate the ones obtained with stdDNA solutions since the amount of DNA eluted using the initial protocol was higher. 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). The type

of matrix has a big influence on the extraction process and the results demonstrated that more tests are required in order to improve the DNA extraction from sesame seeds using these microfluidic systems.

With the prototype 2, the extraction time was reduced by half when compared to the prototype 1. The tests performed with this system demonstrated that, when transferring a protocol defined for the prototype 1 to the prototype 2, there are other features that must be taken into consideration besides the reduction of introduced sample and buffer volumes, namely the residence time. In the smaller system (prototype 2), the fluid passed through the membrane more rapidly because the volume capacity of its chamber is lower, resulting in an inefficient binding and elution steps, which suggested that higher incubation times should be included in these steps or lower flow rates should be selected for further experiments.

The results of the test with the two types of silica membrane demonstrated that the DNA extraction using membrane I was more efficient than using membrane II for both alternatives III and IV. The differences between the membranes using the alternative III was not very big, suggesting that a higher thickness may compensate a higher pore size. However, the DNA extraction yield obtained using the alternative 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.

It is important to refer that the Quant-iT™ PicoGreen® dsDNA assay kit used for quantification required a calibration curve, as described before, and the slopes of these standard curves varied from day to day even using the same calibration standards every time, which might 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 and, therefore, the quantification results of samples with chaotropic salts in solution may be underestimated. In spite of this, the quantification of elution samples and, consequently, the elution yields should not be affected by this limitation because the elution buffers do not contribute for this effect.

In conclusion, the optimization of each phase of a DNA analysis procedure separately allows a better understanding of the features that affect them when performed at microscale. This work described a modular approach for the development and optimization of a DNA extraction protocol based on μ SPÉ, suitable for integration in a microfluidic setting. Although more tests are required to improve the DNA purity and the extraction efficiency when using food samples, the presented protocol optimized in the prototype 1 proved to be suitable for this purpose.

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