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Grau de Química

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Separation and characterization of microRNAs by capillary electrophoresis with ultraviolet detection and mass spectrometry.

Separació i caracterització de microARNs amb electroforesi capil·lar amb detecció ultraviolada i espectrometria de masses.

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"Divideix les dificultats que examines en tantes parts com sigui possible per a la seva millor solució"

René Descartes

Voldria començar donant les gràcies al doctor Fernando Benavente i al doctorant Roger Peró per formar-me sobre les diferents tècniques que s'utilitzen en aquest treball, dirigir-me i orientar-me durant els moments més complicats i ajudar-me amb tots els dubtes que m'han anat sorgint durant el seu transcurs. Donar-li gràcies també a ells i la resta dels membres del grup de bioanàlisi per l'ambient acollidor que generaven (en especial a la Laura i la Montse) i per la seva paciència davant les meves preguntes. M'agradaria dir també que ha estat un plaer treballar juntament amb altres estudiants de TFG i estudiants estrangers que han anat arribant durant el transcurs del treball. Finalment moltes gràcies també a la meva família pel seu suport al llarg de tot el grau, especialment en els moments més durs.

REPORT

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1. SUMMARY

MicroRNAs (miRNA) are a class of small endogenous RNA molecules of about 19-23 nucleotides that act as regulators of genes. Aberrant expression of these oligonucleotides has been associated with a plethora of diseases ranging from nervous system diseases, heart diseases, inherited diseases and different types of cancer. The increasing association between miRNA malfunction and diseases coupled with the advances in detection and characterization of these kinds of analytes have turned miRNAs into an interesting topic of study, especially within the molecular biology field. Due to their stability towards pH, temperature and other factors, applications of microRNAs in forensic studies have also been studied.

In this work we have optimized a capillary electrophoresis with ultraviolet detection method (CE-UV) that allows separating a mixture of synthetic miRNAs with similar nucleotide sequence and length. Compatibility with on-line mass spectrometry detection was also considered during the optimization. The method was validated in terms of repeatability of migration times and peak heights, linearity and limits of detection (LOD).

For characterization of miRNAs matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was used, where mass accuracy and other quality parameters were evaluated and compared between different modes. Better LOD than the CE-UV method and good mass accuracy were achieved using one of the miRNAs as an internal standard for the calibration, though loss of repeatability of peak area and height was observed, making this method unsuitable for quantitative analysis. Sequencing of microRNAs with the tandem mass spectrometry (MS/MS) mode was discarded due to instrument limitations.

Keywords: Capillary electrophoresis, MALDI-TOF-MS, Mass spectrometry, MicroRNA, Optimization, Separation.

2. RESUM

Els microARNs (miARN) són una classe de molècules petites de ARN endògenes d'aproximadament 19-23 nucleòtids que actuen com a reguladors de gens. L'expressió aberrant d'aquest oligonucleòtids s'ha associat amb multitud de malalties, des de malalties del sistema nerviós, del cor, hereditàries i diferents tipus de càncer. L'increment de les associacions entre el funcionament defectuós dels miARN i les malalties, juntament amb els avanços en detecció i caracterització d'aquests tipus d'analits han convertit els miARNs en una interesant temàtica, especialment dins del camp de la biologia molecular. També s'ha estudiat les aplicacions dels miARNs en estudis forenses degut a la seva estabilitat enfront el pH, la temperatura i altres factors.

En aquest treball s'ha optimitzat un mètode d'electroforesi capil·lar amb detecció ultraviolada (CE-UV) que ens permet separar una mescla de miARNs sintètics amb seqüència i longitud de nucleòtid similar. Compatibilitat amb espectrometria de masses en línia també es va considerar durant l'optimització. El mètode es va validar en termes de repetibilitat dels temps de migració i altura de pics, linealitat i límits de detecció (LOD).

Per caracteritzar els miARNs es va utilitzar una tècnica anomenada MALDI-TOF-MS que es denomina per les seves sigles com a espectròmetre de masses amb desorció/ionització de làser assistida per matriu amb analitzador de temps de vol, on l'exactitud de la massa i altres paràmetres es van avaluar i comparar entre diferents modes. Es van aconseguir millors LOD que amb el mètode CE-UV i bona exactitud de la massa utilitzant un dels miARNs com a estàndard intern pel calibratge, però també es va observar una pèrdua de repetibilitat en l'àrea i altura dels pics, indicant que aquest mètode no és adequat per a l'anàlisi quantitatiu. Seqüenciació dels miARNs amb el mode d'espectrometria de masses en tàndem (MS/MS) es va descartar degut a limitacions de l'instrument.

Paraules clau: Electroforesi capil·lar, MALDI-TOF-MS, Espectrometria de masses, MicroARN, Optimització, Separació.

3. INTRODUCTION

3.1. MICRORNAS

MicroRNAs (also known as miRNA or miR for short) are a type of small non-coding ribonucleic acids found in a plethora of organisms, ranging from viruses to plants and animals. These small RNA molecules are around 19-23 nucleotides (adenine A, cytosine C, guanine G and uracil U) long with a 5'-end (phosphate group) and a 3'-end (hydroxyl group) that can present post-transcriptional modifications such as 5'-end dephosphorylation, or other unexpected isoforms with differing ends due to trimming or nucleotide additions [1]. MiRBase is a public database of published miRNA sequences and annotations (www.mirbase.org, [2]).

The first miRNA was discovered in the early 90s [3] but they were not recognized as biological regulators until the 2000s [4]. Recent studies have shown the implication between aberrant miRNA expression and a plethora of diseases, such as inherited diseases, different types of cancer, heart and kidney diseases and even some mental diseases. A publicly available database known as miR2Disease allows the user to search all known relations between aberrant miRNA expression and diseases (www.mir2disease.org, [5]). Due to their high stability, miRNAs have also shown potential in other fields such as biomarkers for forensic body fluid identification [6].

miRNAs are first transcribed from a gene that codifies them, generating a long RNA molecule (primary miRNA or pri-miR) that forms base pairs with itself and folds over to make a hairpin, which is then cut by enzymes resulting in a small double-stranded fragment of around 19-23 nucleotides on both sides. One of the strands of this fragment (mature miRNA) then binds to a specific protein to make an RNA-protein complex and is then directed to "matching" messengerRNA (mRNA) molecules, forming base pairs with the miRNA. When the RNA-protein complex binds with the mRNA different types of inhibition occur, the most typical ones are (Figure 1):

- The miRNA matches perfectly with its target and an enzyme of the complex slices the mRNA in half, leading to its breakdown.

- The miRNA has some mismatches with its target and the complex binds to the mRNA and prevents translation.

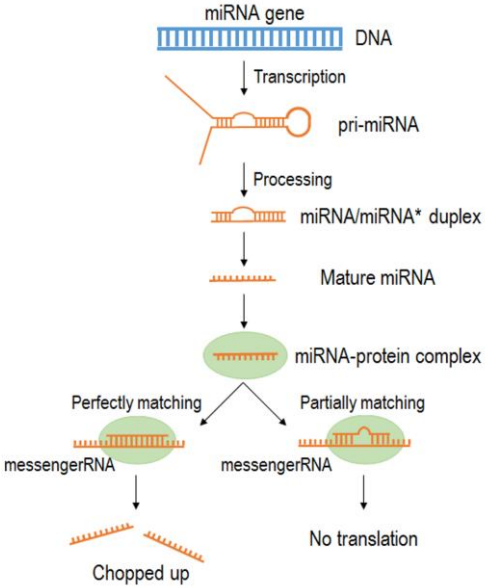


Figure 1. Diagram of miRNA biogenesis and gene regulation.

Inhibition of mRNAs leads to regulation of the proteins they codify. As such, miRNAs are considered gene inhibitors [1]. MiRNAs are found mainly within the cell but some are also found in extracellular environments such as blood, urine or saliva, and are known as extracellular or circulating miRNAs. These miRNAs are packaged in extracellular vesicles and have high stability and resistance to pH, temperature and endogenous RNase activity [7].

3.2. ANALYSIS OF MIRNAS

The methods mostly used for miRNA analysis currently are Northern blotting [8], microarrays [9] and real time quantitative polymerase chain reaction (RT-qPCR) [10]. The last method mentioned is especially used due to its greater sensitivity but has the drawback of being a targeted method, meaning that knowledge of the miRNAs desired for analysis must be known beforehand. Another disadvantage is being an indirect method, meaning that detection is done through detection of complementary DNA instead of the original miRNA. These drawbacks are especially important since miRNAs may undergo modifications after transcription which has

been reported to affect miRNA stability as well as being a mechanism for the regulation of miRNA activity. A diagram of the RT-qPCR can be seen below (Figure 2).

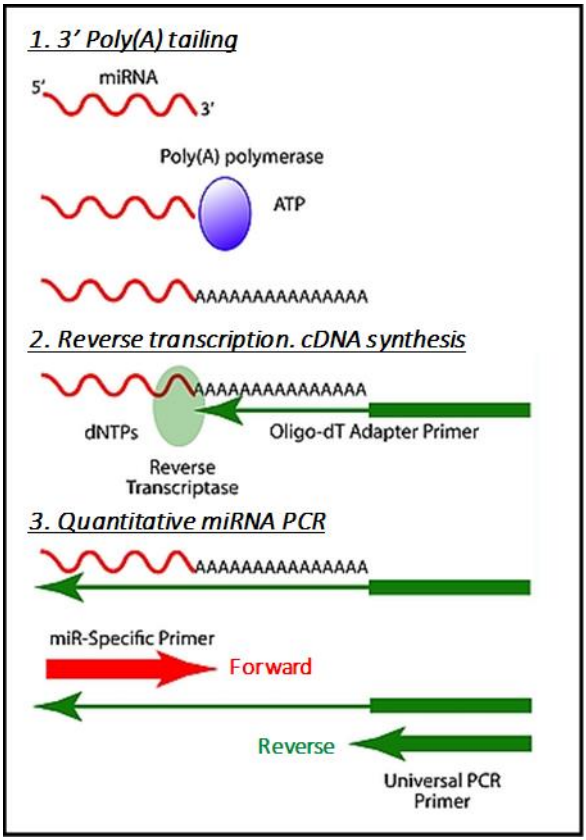


Figure 2. Diagram of the RT-qPCR.

3.3. CAPILLARY ELECTROPHORESIS

Due to miRNAs having similar nucleotide length, charge and structure separation of these types of molecules can be quite difficult by liquid chromatography (LC) or capillary electrophoresis (CE). Capillary zone electrophoresis (CZE) is a highly efficient and very simple electroseparation mode and could be appropriate to separate these polar and negatively charged molecules. Furthermore, using appropriate background electrolytes (BGE), it is possible to use on-line mass spectrometry detection (CE-MS). While not compatible with mass

spectrometry, other electroseparation modes such as capillary gel electrophoresis (CGE) have also been considered for miRNA separation [11].

A typical CE instrument consists of both an inlet and an outlet reservoir both in contact with the anode and the cathode full of BGE that acts as the conductive medium. These reservoirs are connected with a fused-silica capillary that has been filled with the same BGE, where the sample is subsequently loaded inside and injected from the inlet either by using pressure (hydrodynamic injection) or by applying an electric field (electrokinetic injection). Once the sample has been injected an electric field is applied and analytes migrate towards the detector that is near the outlet end (UV detection) or in the outlet end of the capillary (MS detection).

In CE the analytes are separated by the difference in solute velocity in an electric field (Equation 1):

$$v = \mu_e \times E \quad \text{(Eq.1)}$$

Where v stands for ion velocity, μ_e stands for electrophoretic mobility and E is the electrical field applied. μ_e of an analyte is constant for a given ion and medium and is determined by the electric force that the ionized species experiences and its frictional drag through the medium. The charged species move towards the electrode of the opposite charge described by the charge-to-ionic radius ratio (q/r), as consequence of that, electrophoretic mobility is very dependent on the BGE composition and pH. However, there is another factor that affects the mobility of these ions called electroosmotic flow (EOF), which is the consequence of the negatively charged silanol groups that are found inside the capillary wall of the fused-silica capillary. Due to that, a double-layer is formed with counter-ions (cations in this case) near the surface to maintain charge balance and is then attracted towards the cathode whenever voltage is applied. Because our analytes are solvated, the movement of the double-layer drags our solution towards the cathode, where both positive neutral and negative ions will be detected. Positive ions will be detected faster due to having both electrophoretic and EOF towards the cathode, neutral ions will be detected all at the same time due to not having different mobility (they require charge in order to be separated) and finally negative ions will be detected if the EOF mobility is bigger than the μ_e. Since EOF is a consequence of the deprotonated silanol groups inside the capillary wall, pH and good maintenance of the capillary wall is essential for good reproducibility of EOF.

CZE is the preferred mode to optimize a method potentially compatible with CE-MS, which requires the use of volatile BGEs for good electrospray ionization (ESI) and on-line mass spectrometry detection [7]. However, it is not the preferred mode for separation of oligonucleotides, miRNAs or DNA.

The development of gel electrophoresis of nucleic acids in 1977 helped develop groundbreaking sequencing technologies that allowed visualization of DNA fragments via radioactive labelling combined with gel electrophoresis [12]. CGE was crucial in the human genome project [13] and the method of choice before the emergence of next generation sequencing techniques (NGS) [14]. In CGE a gel is added to the BGE, where separation is based on the difference in analyte size as they migrate through the gel, meaning that the less impeded miRNAs will be detected faster. Since gels are a high viscosity medium, they also limit heat transfer by slowing down the molecules, reducing the effects of Joule heating. Since the frictional drag increases a lot, it is possible that negatively charge ions cannot be detected in normal polarity. In those cases, reversing the polarity is essential for analyte detection with the drawback that analytes may arrive too fast to the detector and not separate enough. Suppression of EOF can allows us to separate these miRNAs in reverse polarity, which can be achieved using capillary coatings. Since this technique separates analytes by size neutral analytes can also be separated if their sizes are different enough [13].

Micellar electrokinetic chromatography (MEKC) was also considered for miRNA analysis. In this mode separation of analytes is based on the partitioning of analytes between the micelles that form when a surfactant is added to the BGE above the critical micelle concentration (CMC) and the solvent. Depending on the surfactant used micelles can be charged positively or negatively. The difference in hydrophobicity of our analytes will make it so that the most hydrophobic will spend more time in the micelle and will have different mobility compared to the ones that spent more time travelling through the solvent. This mode is mainly used for separation of neutral compounds but can also be used to separate similar compounds such as miRNAs [13].

3.4. MALDI-TOF-MS

Since miRNAs can present post-transcriptional modifications that cannot be detected with the analytical methods typically used to analyse them a good characterization method based on mass spectrometry (MS) is highly recommended. Mass spectrometry is a good candidate since it allows us to detect ions by their mass-to-charge ratio (m/z), meaning that with enough accuracy we can differentiate between very similar miRNAs isoforms. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has been used mainly for proteomics research and more recently for the genomic research, specifically in the analysis of oligonucleotides [15]. This soft ionization technique is useful for the characterization of oligonucleotides since it promotes minimal fragmentation (like ESI) of the target analytes while allowing high ionization of thermolabile non-volatile analytes [16], having also high resolution and easier to interpret mass spectra compared to ESI due to generating fewer multicharged ions. Sequencing studies of oligonucleotides including miRNAs have been performed with MALDI-TOF/TOF-MS [17].

In order to perform the analysis, analytes are crystalized alongside a small organic compound also known as “matrix”. The mixture is then irradiated with an UV laser, where matrix molecules are excited and analyte molecules undergo ion desorption on contact with minimal fragmentation. Additives can also be included in the matrix, such as ammonium citrate, to supress the formation of salt adducts, such as sodium and potassium adducts [15].

Once the analyte ions are generated, they are then drawn towards the time-of-flight (TOF) mass analyser, where ions are accelerated with a uniform electromagnetic force towards a flight tube of about 1-2 meters. Due to this uniform electromagnetic force, all ions have the same kinetic energy, where lighter ions travel faster compared to the heavier ones and arrive to the detector at different times. The m/z ratio of ions is determined by their arrival time to the detector.

MALDI-TOF-MS analysis can be done with different modes, depending on how the ions travel towards the detector [17]:

- In the linear ion detector mode ions travel a linear path towards the detector.
- In the reflector ion detector mode, the direction of travel of the ions is reversed with an ion mirror. Using this method, we can diminish substantially the spread of flight times

of ions with the same mass-to-charge ratio, allowing isotopic distribution peaks or other subtle differences to be resolved.

- In the MS/MS ion detector mode the collision of gas molecules with the parent ions induces further ion fragmentation which are then subjected to a second TOF mass analyser and resolved.

4. OBJECTIVES

In this work, the main objective is to accomplish separation and characterization of a standard mixture of miRNAs which are relevant biomarkers using capillary electrophoresis with ultraviolet detection (CE-UV) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

The specific objectives of this work are:

- Optimization of a reproducible CE-UV method, having high enough resolution to differentiate our miRNAs.
- Compatibility of the CE-UV method with on-line MS detection.
- Evaluation of the quality parameters of the optimized CE-UV method, where repeatability of migration times and peak height, linearity and limits of detection (LODs) are established.
- Optimization of a reproducible MALDI-TOF-MS method for characterization of our miRNAs.
- Evaluation of quality parameters of MALDI-TOF-MS, where accuracy, repeatability on ion signal, linearity and LODs are established.

5. EXPERIMENTAL SECTION

5.1. INSTRUMENTATION

CE-UV experiments were performed with a 7100 CE instrument (Agilent technologies, Waldbronn, Germany) equipped with a photodiode array detector (DAD). Results were subsequently processed with OpenLab CDS C.01.07 SR3 ChemStation edition software (Agilent technologies).

Characterization of miRNAs was done with a 4800 MALDI TOF/TOF mass spectrometer (Applied Biosystems, Waltham, MA, USA) equipped with a neodymium-doped yttrium aluminum garnet laser (Nd: YAG, 355nm) and a microchannel plate detector. Data acquisition and data processing were performed using 4800 Series Explorer™ and Data Explorer Version 4.5 software (Applied Biosystems). Laser intensity of 6500 units and linear mid mass negative mode were selected as the best conditions for miRNA characterization. The miRNA hsa-let-7g-5p was selected as internal standard for increased mass accuracy.

5.2. SAMPLES

All synthetic miRNAs were purchased from Integrated DNA technologies (Coralville, Iowa, United States) with desalting as the purification method. Table 1 shows the miRNAs used in this study along with some other relevant information. These solid miRNAs where then dissolved with water of LC-MS grade (Fisher) until 100µM concentrations were achieved. These solutions were aliquoted in multiple Eppendorf's vials (0.5mL of capacity) and stored inside in freezer at -4ºC. Consecutive freeze and thaw cycles were discouraged due to oligonucleotide degradation. Once unfrozen standard solutions were stored inside a fridge at 2-8ºC for subsequent uses. All mixtures were prepared from the stock standard solutions and vortexed before usage. Due to degradation diluted mixtures were discarded after 1 day of usage (concentrated mixtures didn't have this problem and could be stored for at least 1 week in the fridge at 2-8ºC).

Number	miRNA ID	Sequence	Accession number[c]	Length [nt]
1	hsa-miR-223-3p[a]	ugucaguuugucaaauacccca[a]	MIMAT0000280	22
2	hsa-miR-21-5p	uagcuuaucagacugauguuga	MIMAT0000076	22
3	hsa-let-7a-5p	ugagguaguagguuguauaguu	MIMAT0000062	22
4	hsa-let-7g-5p	ugagguaguaguuuguacaguu	MIMAT0000414	22
5	hsa-miR-16-5p+3'U[b]	uagcagcacguaaauauuggcgu[b]	MIMAT0000069[d]	23

(a) This miRNA sequence goes from 3' to 5'. All other miRNA sequences go from 5' to 3'.
(b) This miRNA has an added nucleotide (U) in its 3' end and is also known as isomiR16.
(c) Accession number of mature miRNAs from miRbase [2], where information about miRNA sequence and other related things were found.
(d) Accession number of hsa-miR16-5p, since the isoform couldn't be found inside the database.

Table 1. List of synthetic miRNAs used, their sequence, accession number and nucleotide length.

5.3. CHEMICALS AND REAGENTS

5.3.1. Chemicals and reagents of the CE-UV method

All chemicals used for CE-UV were of analytical reagent grade. Ammonium acetate (NH_4Ac, Sigma-Aldrich), ammonium bicarbonate (NH_4HCO_3, Fluka), boric acid (H_3BO_3, Merck), tris(hydroxymethyl)aminomethane (Tris, J.T. Baker) and sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$, Merck) were selected as BGE candidates.

Methanol for UHPLC Supergradient (MeOH, ACS), acetonitrile of LC-MS grade (ACN, PanReac), heptakis-(2,3-dimethyl-6-sulfato)-β-cyclodextrin sodium salt (HDMS-βCD, Regis Technologies), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Sigma-Aldrich), triethylamine (TEA, Baker) and perfluorooctanoic acid (PFOA, Sigma-Aldrich) were tested as BGE additives.

A 1/8 (v/v) diluted ammonia (NH_3) solution prepared from a 25% NH_3 concentrated solution (Merck) and a 1M solution of hydrochloric acid (HCl) prepared from a 25% HCl concentrated solution (Merck) were used to adjust BGE pH. All BGE solutions were prepared and adjusted to pH9 using the potentiometer Sension+ PH3 (HACH-LANGE, Hospitalet, Barcelona) and stored inside a fridge until usage was required.

Before usage all BGE solutions were sonicated in an ultrasounds bath (P-selecta, Barcelona, Spain) for 5 minutes and filtered with polyamide (Nylon) membrane filters of 0.20 pore size (Macherey-Nagel).

A potassium hydroxide (KOH) 1M solution dissolved in MeOH was prepared as part of the capillary coating protocol for CGE. Poly (vinyl alcohol) of 89-98kDa hydrolysed (PVA, Sigma-Aldrich) and a 50% polyethylene glycol (PEG) solution of 7000-9000Da were added to the BGE as additives for CGE.

All previously mentioned solutions were prepared with ultrapure water with a conductivity that has less than 0.05 S/Cm, obtained from the water purification system Synergy® (Merck, Darmstadt, Germany). The analytical balance Mettler-Toledo AT261 DeltaRange (Mettler Toledo Ag, Schwerzenbach, Switzerland) was used to weigh all the solids.

Due to the multitude of chemicals and different modes that will be used, CE electrodes were cleaned whenever different reagents were used. This cleaning process consist of submerging both electrodes in a mixture of 50% 2-propanol HPLC grade (iPrOH, PanReac) and 50% of ultrapure water and sonicate the solutions for 5 minutes with an ultrasound bath.

5.3.2. Chemicals and reagents of the MALDI-TOF-MS method

The matrix used to prepare the spots in the MALDI-TOF-MS technique was composed of 2',4',6'-Trihydroxyacetophenone monohydrate (THAP, Fluka) and ammonium citrate dibasic (AC, Fluka). A solution of THAP of 50mg/mL was prepared with H_2O LC-MS grade (Fisher):ACN LC-MS grade (PanReac) at 1:1, same concentration as AC but with only H_2O LC-MS. 5µL were pipetted from sample, mixed and vortexed with 5µL of AC inside an Eppendorf's vial. From this mixture 5µL were pipetted, mixed and vortexed with 5µL of the THAP solution. From here, 3 spots of 1µL were prepared on a plate for subsequent MALDI-TOF-MS analysis.

Before analysis a nitrogen gas current was applied on the plate to ensure dryness of the sample and to discard any dust accumulated on top of the spot.

5.4. EXPERIMENTAL PROCEDURES

5.4.1. Optimized conditions of the CE-UV method

New capillaries were activated flushing (930mbar) with the following solutions:

1. 10 min H_2O
2. 10 min HCl 1M
3. 10 min H_2O
4. 10 min NaOH 1M
5. 10 min BGE

Between workdays conditioning consisted in:

1. 10 min H_2O
2. 10 min NaOH 1M
3. 10 min H_2O
4. 10 min BGE

The optimized conditions for analysis were:

1. Preconditioning: Flush with BGE for 5 min.
2. Analysis:
   - Injection: Hydrodynamic injection, 50mbar 10s.
   - Temperature: 10ºC of cassette and sample tray.
   - Voltage: 15kV (positive polarity).
   - Analysis time: 20min.
   - Capillary: Fused-silica capillaries (Polymicro Technologies) of 50µm internal diameter (i.d.) x 57cm total length (L_T) x 360µm outer diameter (o.d.) were selected as the best capillaries for miRNA separation by CZE. The detection window was placed at 50cm from the inlet of the capillary. A fused-silica capillary (Polymicro Technologies) of 75µm i.d. x 44cm L_T x 360µm o.d. coated with PVA (89-98kDa) was used for CGE with the detection window placed at 37cm from the inlet of the capillary.
   - Detection wavelengths: 200 and 260nm.
3. Postconditioning: Flush with water for 2 min.

5.4.2. Capillary coating protocol for CGE

A capillary coating with thermally stabilized polyvinyl alcohol (PVA) was selected for CGE analysis [18] [19]. This protocol consists of the following steps:

1. A 5% (w/v) solution of PVA 89-98kDa (99% hydrolysed, Sigma-Aldrich) was prepared by weighting the solid PVA inside an Eppendorf's vial, dissolving with water (ultrapure) and heating in a Thermo-Shaker TS-100 (Biosan, Riga, Latvia) at 80ºC for 1 hour. Solution must be kept at 80ºC until usage.
2. Three capillaries with 60cm or more of length were cut to prepare several at the same time, which could be useful in case the capillaries get clogged. Internal diameter of capillaries must be 75µm to prevent clogging.
3. The capillary was coiled so that 10cm stick out from the coil at each end.
4. The capillary was then conditioned using the following steps:
   - 5 min H_2O
   - 10 min MeOH
   - 10 min KOH 1M dissolved in MeOH
   - 10 min H_2O
   - 10 min HCl 1M
5. Once conditioned the coiled capillary was placed inside an oven, heating at 140ºC for 60min. Temperatures above 160ºC must be avoided as they may thermally degrade the polymer within the capillary. The holes exiting from the oven are then sealed with a septum. The ends of the capillary were placed inside vials, one end containing the PVA solution and the other water. The PVA vial was inside a Kitasato flask which was then sealed with a rubber cap that allowed the capillary to pass through it. Nitrogen (N_2) was then flushed at 0.7 bars for 1 hour. A clamp was used to prevent the rubber cap from opening. This setup can be seen in Figure 3.

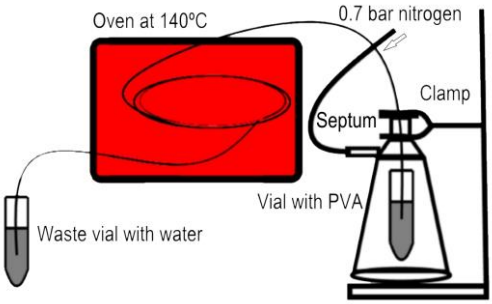


Figure 3. Set-up of the capillary coating protocol.

6. The capillary was flushed with PVA for 1 hour. Then the vial with PVA was removed and N_2 was flushed at 0.7 bars for 1 hour or until bubbles of N_2 are seen in the waste vial of water. If no bubbles were seen after 1 hour 0.5 cm of the inlet and the outlet capillary were removed, discarding the clogged parts and the flush with N_2 was repeated.

5.4.3. Optimized conditions of the MALDI-TOF-MS method

To obtain high mass accuracy of miRNAs by MALDI-TOF-MS different parameters were checked and optimized:

- Laser intensity: Set to 6500.
- Modes: Linear mid mass negative.
- Calibration: The miRNA hsa-let-7g-5p was used as an internal standard for calibration before sample analysis.

6. RESULTS AND DISCUSSIONS

6.1. OPTIMIZATION OF THE CE-UV METHOD

Optimization of the CE-UV method was organized into three different groups: parameters related to the BGE (such as pH, additives and ionic strength), instrumental parameters (such as temperature, voltage, capillary type, injection conditions and selection of detection wavelength) and CGE conditions (separated from the other steps due to difference of instrumentation and materials). Once optimized several quality parameters of the method were studied.

6.1.1. BGE optimization

Five different BGEs were prepared as candidates for miRNA separation; containing: NH_4Ac [7], NH_4HCO_3 [20], H_3BO_3 [21], NaH_2PO_4 [22] and Tris [13]. These BGEs were selected for their reproducible EOF, similar ionic strength, low cost and good results with oligonucleotide separations [13]. NH_4Ac and NH_4HCO_3 were of special interest due to their potential compatibility with on-line mass spectrometry detection because they were more volatile than the rest. The conditions of the different BGEs tested and electropherograms with the results obtained for a mixture of two miRNAs (hsa-miR-223-3p and hsa-let-7a-5p) can be seen below (Table 2, Figures 4, 5, 6, 7 and 8).

BGE	Concentration [mM]	pH[a]	Ionic strength [mM][b]	Buffer index[c] [mol · L^{-1} · pH^{-1}]
NH_4Ac	25	9.02	25mM	0.020
NH_4HCO_3	20	9.58	29mM	0.045
H_3BO_3	60	9.02	25mM	0.034
NaH_2PO_4	10	7.96	23mM	0.002
Tris	250	9.09	25mM	0.052

(a) pHs were calculated with CurTiPot version 4.2.0 (Ivano G. R. Gutz, São Paulo, SP, Brasil). All ammonium buffers were adjusted with diluted ammonia solutions, all the other buffers except Tris were adjusted with NaOH 1M. Tris solution was adjusted with HCl 1M.

(b) Ionic strength was also calculated with CurTiPot.

(c) Buffer index, also known as buffer capacity is the amount of strong acid/base needed to shift pH by one unit, though the estimates are only reasonably accurate when talking about 0.1 units of difference. Buffer capacity is important since BGE composition may vary under high voltages.

Table 2. BGE conditions.

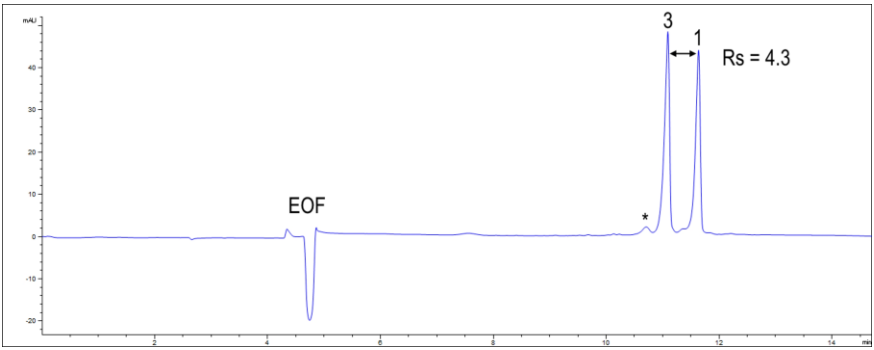


Figure 4. CE-UV electropherogram. BGE: NH_4Ac 25mM pH9. Capillary: 75µm i.d. x 57cm L_T x 360µm o.d.. Instrument conditions: 15kV (voltage), 200nm (wavelength), 25ºC (cassette), 10ºC (tray) and hydrodynamic injection 50mbar 10s. Sample: hsa-miR-223-3p (1) + hsa-let-7a-5p (3) in water at 10µM. R_s=4.3

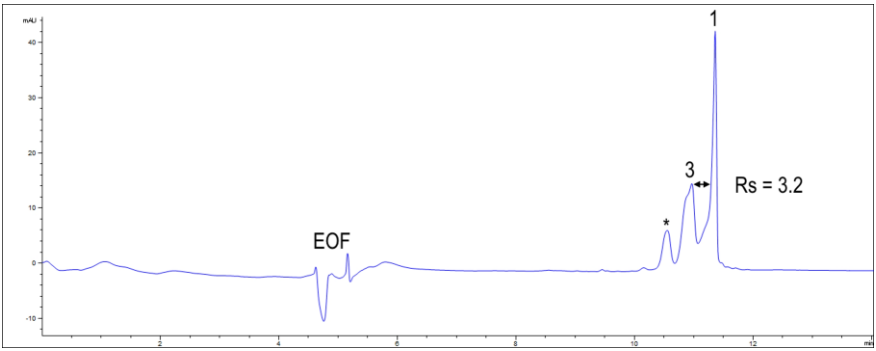


Figure 5. CE-UV electropherogram. BGE: NH_4HCO_3 20mM pH9.6. Capillary: 75µm i.d. x 57cm L_T x 360µm o.d.. Instrument conditions: 15kV (voltage), 200nm (wavelength), 25ºC (cassette), 10ºC (tray) and hydrodynamic injection 50mbar 10s. Sample: hsa-miR-223-3p (1) + hsa-let-7a-5p (3) in water at 10µM. R_s=3.2

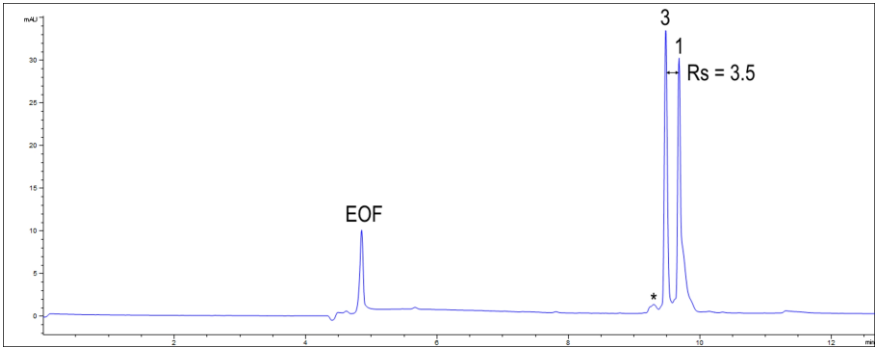


Figure 6. CE-UV electropherogram. BGE: H_3BO_3 60mM pH9. Capillary: 75µm i.d. x 57cm L_T x 360µm o.d.. Instrument conditions: 15kV (voltage), 200nm (wavelength), 25ºC (cassette), 10ºC (tray) and hydrodynamic injection 50mbar 10s. Sample: hsa-miR-223-3p (1) + hsa-let-7a-5p (3) in water at 10µM. R_s=3.5

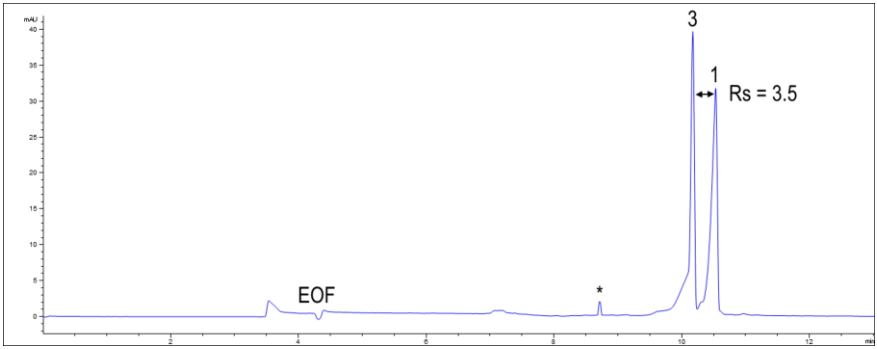


Figure 7. CE-UV electropherogram. BGE: NaH_2PO_4 10mM pH8. Capillary: 75µm i.d. x 57cm L_T x 360µm o.d.. Instrument conditions: 15kV (voltage), 200nm (wavelength), 25ºC (cassette), 10ºC (tray) and hydrodynamic injection 50mbar 10s. Sample: hsa-miR-223-3p (1) + hsa-let-7a-5p (3) in water at 10µM. R_s=3.5

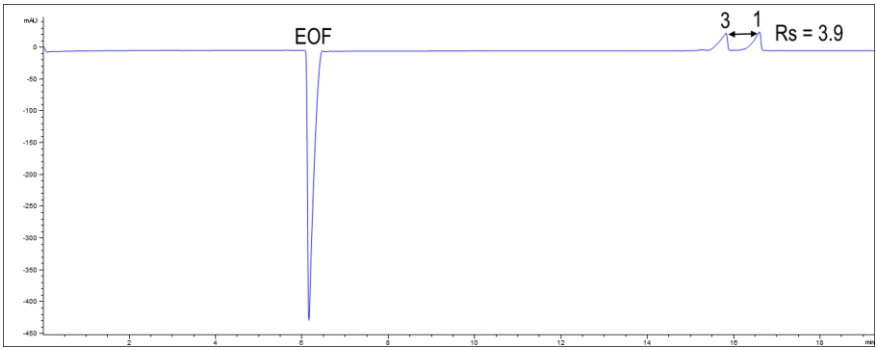


Figure 8. CE-UV electropherogram. BGE: Tris 250mM pH9. Capillary: 75µm i.d. x 57cm L_T x 360µm o.d.. Instrument conditions: 15kV (voltage), 200nm (wavelength), 25ºC (cassette), 10ºC (tray) and hydrodynamic injection 50mbar 10s. Sample: hsa-miR-223-3p (1) + hsa-let-7a-5p (3) in water at 10µM. R_s=3.9

Using separation voltages between 5 and 25 kV at 25ºC all the BGEs followed the Ohm's law. At these pH values the miRNAs were negatively charged and eluted after the negative (positive with H_3BO_3) dip due to the EOF. Several impurities were also detected (indicated with *), which intensity increased with sample aging. The total analysis time was between 10 min (H_3BO_3 BGE) and 17 min (Tris BGE).

Peak resolution (R_s) was calculated following Equation 2:

$$R_s = \frac{1}{4} \times N^{\frac{1}{2}} \times \left(\frac{\Delta\mu_a}{\overline{\mu_a}}\right) \quad \text{(Eq.2)}$$

Where resolution of two components is expressed with respect to efficiency. $\Delta\mu_a$ stands for the difference between electrophoretic mobility of two analytes, $\overline{\mu_a}$ stands for the average electrophoretic mobility between those analytes and N stands for theoretical plate number. It should be noted that this formula considers the peaks to be gaussian, meaning that electropherograms with heavy peak asymmetry may present higher resolution than expected. Theoretical plate number for gaussian peaks can be calculated using Equation 3:

$$N = 5.54 \times \frac{t}{w_{1/2}} \quad \text{(Eq.3)}$$

Apparent electrophoretic mobility was calculated using Equation 4:

$$\mu_{ap} = \frac{I \times L}{t \times V} \quad \text{(Eq.4)}$$

Where I stands for effective capillary length to detector (cm), L stands for total capillary length (cm), t is migration time and V stands for applied voltage (in V). Subtracting electrophoretic mobility of EOF (using the same formula) with the calculated apparent mobilities allows us to find the real electrophoretic mobilities of our analytes that are used for resolution calculations.

As can be observed in the electropherograms most of the BGEs accomplished miRNA separation at baseline (except NH_4HCO_3 due to its non-gaussian peaks). NH_4Ac was selected as the best BGE for separation due to having the highest resolution (R_s=4.3) and peak symmetry. Tris BGE presented certain peak asymmetry with fronting in its peaks, which might be an indicator of the difference in mobilities between the sample ions and the BGE electrolyte

ions. If the BGE co-ion has lower mobility than that of the analyte fronting peaks occurs [23]. Delayed migration times were also present in subsequent analysis for Tris BGE.

The NH_4Ac BGE was adjusted to pH9 and a new miRNA was added to the mixture (hsa-miR-21-5p). Higher pHs were tested but separation was worse as shown in Figure 9 at pH9.6 [R_s=4.3 (pH 9, Figure 4) > R_s=2.8 (pH 9.6, Figure 9)].

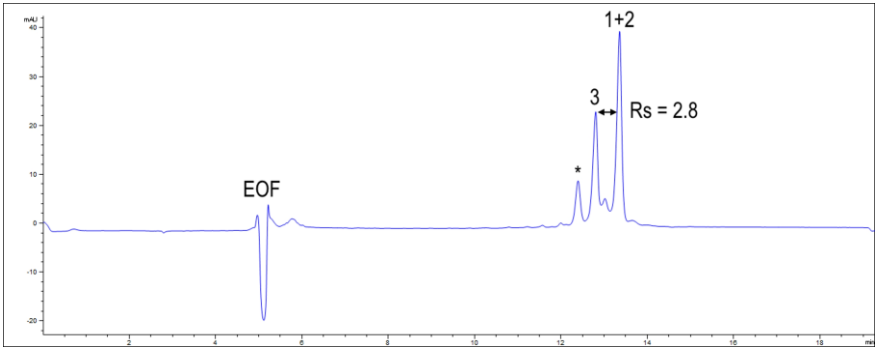


Figure 9. CE-UV electropherogram. BGE: NH4Ac 25mM pH9.6. Capillary: 75µm i.d. x 57cm L_T x 360µm o.d.. Instrument conditions: 15kV (voltage), 200nm (wavelength), 25ºC (cassette), 10ºC (tray) and hydrodynamic injection 50mbar 10s. Sample: hsa-miR-223-3p (1) + hsa-let-7a-5p (3) + hsa-miR-21-5p (2) in water at 5µM. R_s=2.8

In regards with ionic strength it was observed to have different effects depending on cassette temperature. A series of experiments were designed to test that. The experimental design and the results obtained will be explained in the instrumental section. The general idea behind ionic strength modification is that higher values yield lower EOF while lower values yield higher EOF. This is a consequence of the double-layer compression [13].

Different additives were added to the 25mM NH_4Ac pH9 BGE to improve separations:

- Methanol (MeOH) and acetonitrile (ACN): Effects of 10% of organic solvent addition on the BGE were tested. The idea behind their usage is the modification of electroosmotic flow and electrophoretic mobility [24]. Migration times increased with solvent addition due to the increase in viscosity and EOF reduction, increasing approximately 2 minutes with ACN and 9 minutes with methanol, though resolution remained mostly unchanged (Figures 10 and 11).

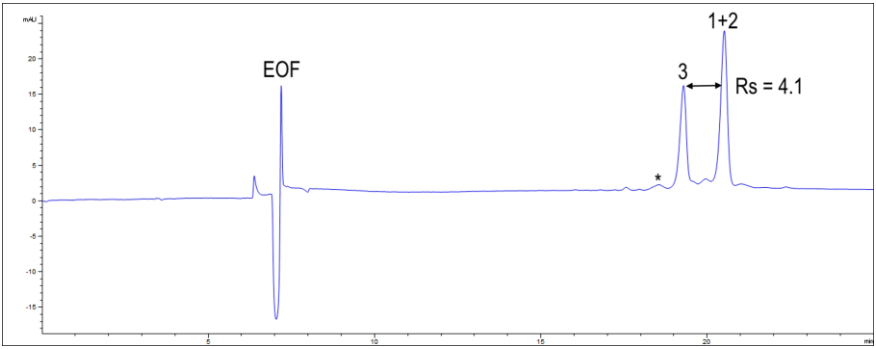


Figure 10. CE-UV electropherogram. BGE: NH4Ac 25mM pH9 + 10%MeOH. Capillary: 75µm i.d. x 57cm L_T x 360µm o.d.. Instrument conditions: 15kV (voltage), 200nm (wavelength), 25ºC (cassette), 10ºC (tray) and hydrodynamic injection 50mbar 10s. Sample: hsa-miR-223-3p (1) + hsa-let-7a-5p (3) + hsa-miR-21-5p (2) in water at 5µM. R_s=4.1

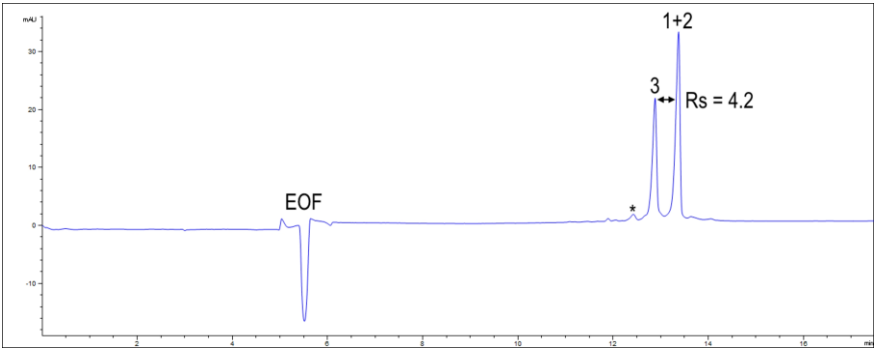


Figure 11. CE-UV electropherogram. BGE: NH4Ac 25mM pH9 + 10%ACN. Capillary: 75µm i.d. x 57cm L_T x 360µm o.d.. Instrument conditions: 15kV (voltage), 200nm (wavelength), 25ºC (cassette), 10ºC (tray) and hydrodynamic injection 50mbar 10s. Sample: hsa-miR-223-3p (1) + hsa-let-7a-5p (3) + hsa-miR-21-5p (2) in water at 5µM. R_{s1}=4.2

- Heptakis-(2,3-dimethyl-6-sulfato)-β-cyclodextrin sodium salt (HDMS-βCD): Concentrations of 2, 5 and 10mM of this cyclodextrin were tested on the BGE. The idea behind the usage of cyclodextrins is that the analytes could have a host-guest complexation with the cyclodextrin cavity, meaning that those with higher affinity will spend more time inside the cyclodextrin, travelling at different velocities compared to the ones with less affinity [13]. An increase of migration times along with loss of repeatability of said times (2-7% RSD) was observed between consecutive analysis. Resolution ranged from 4 to 5.6, where the higher resolutions were obtained from the 10mM solution. This additive was eventually discarded due to the loss of repeatability

between replicates. This loss of repeatability may be because equilibrium between cyclodextrin and analytes was not fully achieved. Another possibility could be due to inner capillary surface modification after cyclodextrin addition. We did not try to solve this repeatability issue because this additive is expensive and non-volatile, hence incompatible with on-line MS detection. Figure 12 shows the best separation achieved through all replicates which was slightly better than without the additive.

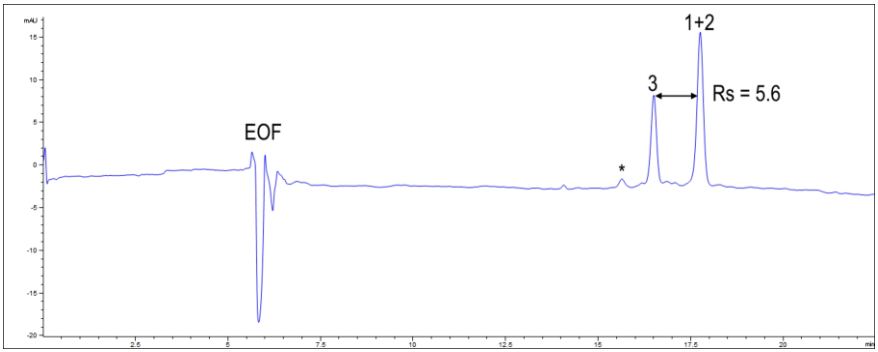


Figure 12. CE-UV electropherogram. BGE: NH4Ac 25mM pH9 + 10mM HDMS-βCD. Capillary: 75µm i.d. x 57cm L_T x 360µm o.d.. Instrument conditions: 15kV (voltage), 200nm (wavelength), 25ºC (cassette), 10ºC (tray) and hydrodynamic injection 50mbar 10s. Sample: hsa-miR-223-3p (1) + hsa-let-7a-5p (3) + hsa-miR-21-5p (2) in water at 5µM. R_s=5.6

- 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) + triethylamine (TEA): Concentrations of 200mM HFIP and 7.5 mM TEA were added to the BGE and a new mixture of 3 miRNAs was used as sample: hsa-miR-21-5p (2), hsa-let-7g-5p (4) and hsa-isomiR-16-5p (5). The idea behind their usage is that TEA forms an ion-pair with negatively charged analytes while HFIP is added as a buffer [25]. Analytes that have higher affinity for forming ion-pairs will travel at different velocities compared to those that have lower ones. A slight increase in migration times and a great loss of resolution was observed (Figure 13).

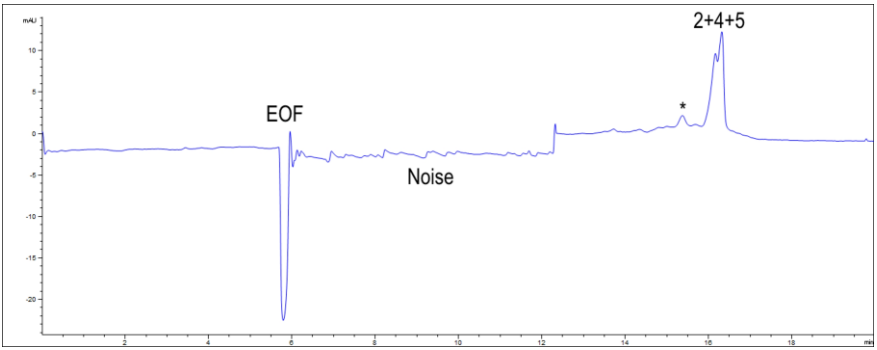


Figure 13. CE-UV electropherogram. BGE: NH4Ac 25mM pH9 + 200mM HFIP + 7.5mM TEA. Capillary: 50µm i.d. x 57cm L_T x 360µm o.d.. Instrument conditions: 15kV (voltage), 200nm (wavelength), 25ºC (cassette), 10ºC (tray) and hydrodynamic injection 50mbar 5s. Sample: hsa-isomiR-16-5p (5) + hsa-let-7g-5p (4) + hsa-miR-21-5p (2) in water at 5µM.

- PFOA: Concentration of 75mM PFOA was added to the BGE. Since PFOA is a surfactant the objective behind its usage is the formation of micelles (MEKC). Analytes that have high hydrophobicity will spend more time travelling inside the micelle and have different electrophoretic mobility compared to the ones that travel more through the solvent [26]. PFOA was eventually discarded due to heavy baseline noise, high current and no detection of miRNA.

No additives were added to the 25mM NH_4Ac BGE under the optimized conditions.

6.1.2. Instrumental parameters

Different instrumental parameters were checked during the course of this work. During optimization of this part the mixtures of less resolved miRNAs hsa-miR-21-5p (2), hsa-let-7g-5p (4) and hsa-isomiR-16-5p (5) (mixture 2) were used.

The different instrument parameters optimized in this work were:

- Wavelength: 200, 210, 214, 230, 250, 260, 273 and 280nm were tested. 200nm was selected as the best wavelength due to EOF peak visibility and highest miRNA S/N ratio. 260nm was also used but had lower S/N ratios and EOF peak was not visible.
- Capillary selection, injection time and injected volume: Capillaries of 50µm i.d. x 57cm L_T x 360µm o.d. and 75µm i.d. x 57cm x 360µm o.d. were tested for miRNA separation. Different internal diameters will inject different volumes of sample in the capillary under the same injection conditions. Equation 5 is used to calculate the injected plug volume:

$$V_{inj} = \frac{\Delta P \times i.d.^4 \times \pi \times t_{inj}}{128 \times \eta \times L} \quad \text{(Eq.5)}$$

Where ΔP is the internal pressure of the injection, i.d. is the internal diameter of the capillary, t_{inj} is the injection time, η is the viscosity of the BGE (considered as 1 for ease of calculations) and L is the capillary length. Injected plug volume can be converted into plug length with the cylinder volume equation (Equation 6):

$$V_{inj} = \pi \times i.d.^2 \times L_{plug} \quad \text{(Eq.6)}$$

Where i.d. stands for internal diameter of the capillary. The %plug length is then calculated with the total capillary length. This %plug length was the criteria used for selecting t_{inj} (Table 3).

P_{inj} [mbar]	t_{inj} [s]	I.D. [µm]	V_{inj} [nL]	L_{plug} [mm]	Plug %[a]
50	5	75	34	7.7	1.4
50	10	50	13	6.9	1.2

(a) Similar plug% were used as the criteria behind injection time selection. Viscosity was considered 1 for ease of calculations.

Table 3. Plug % calculation.

Capillaries of 50µm of i.d. and injection times of 5s were selected as the best conditions for miRNA separation. These capillaries presented an increase in resolution of about 30% while also maintaining migration times. A slight loss of signal was also observed due the smaller volumes of injection. Figures 14 shows an electropherogram of a mixture of 2 miRNAs hsa-miR-21-5p (2) and hsa-let-7g-5p (4) with a 50µm i.d. capillary. This mixture was less resolved than the original, so resolutions appear overall lower.

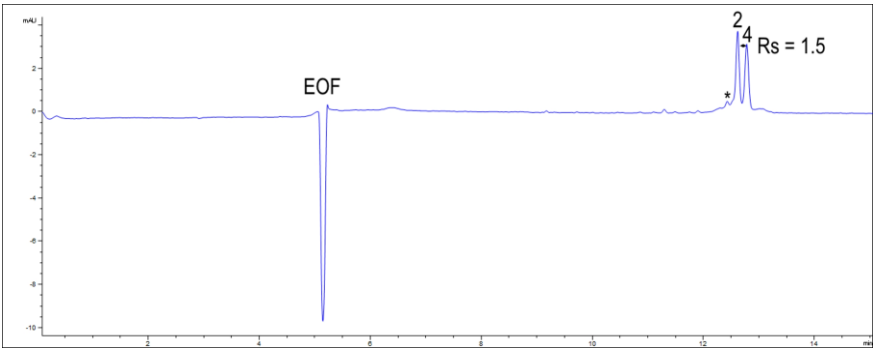


Figure 14. CE-UV electropherogram. BGE: NH4Ac 25mM pH9. Capillary: 50µm i.d. x 57cm L_T x 360µm o.d.. Instrument conditions: 15kV (voltage), 200nm (wavelength), 25ºC (cassette), 10ºC (tray) and hydrodynamic injection 50mbar 5s. Sample: hsa-let-7g-5p (4) + hsa-miR-21-5p (2) in water at 5µM. R_s=1.5

- Voltage: Normal polarity (cathode in the outlet) was used for miRNA analysis with 5, 10, 15, 20 and 25kV. Since ion velocity depends on the electric field an increase or decrease of voltage will affect resolution. However, low voltages yield lower efficiency and resolution and have higher migration times in contrast, high voltages will generally yield lower migration times and higher efficiency, but its effects are often limited due to excessive Joule heating [13]. An overlay of all voltages can be seen on Figure 15. Similar resolutions were obtained for all voltages. 15kV was selected as the best compromise between separation and total analysis time. Under these voltage conditions the Ohm's law was fulfilled as before with a 75µm i.d. capillary.

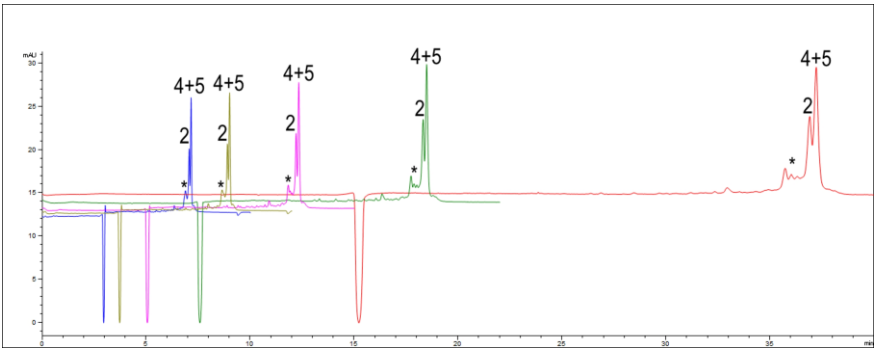


Figure 15. CE-UV electropherogram overlay. BGE: NH4Ac 25mM pH9. Capillary: 50µm i.d. x 57cm L_T x 360µm o.d.. Instrument conditions: 5-25kV (voltage), 200nm (wavelength), 25ºC (cassette), 10ºC (tray) and hydrodynamic injection 50mbar 5s. Sample: hsa-isomiR-16-5p (5) + hsa-let-7g-5p (4) + hsa-miR-21-5p (2) in water at 5µM.

- Cassette temperature and ionic strength: A relation between ionic strength and temperature was observed during the experiments. Viscosity of the BGE changes with temperature, having higher viscosities at lower temperatures [13]. This change in viscosity also changes EOF. Since ionic strength also modifies the EOF a series of experiments was made to test resolution of our analytes under different conditions. BGE concentrations of 10, 25, 50 and 100mM and temperatures of 10, 25, 40 and 55ºC were tested in this 4^2 factorial experimental design, where the lowest resolution between the different miRNAs of the mixture of three was displayed (0 if no separation). Figure 16 shows an electropherogram of the mixture of 3 miRNAs with 10mM NH_4Ac pH9 at 10ºC. Under these conditions the 3 miRNAs were resolved. Results for the different conditions can be found in Table 4. 10mM of BGE concentration and 10ºC of cassette temperature were selected as the best conditions for miRNA separation because R_s was the highest.

Minimal R_s		**T [ºC]**			
		10	**25**	**40**	**55**
[BGE] [mM]	**10**	1.28	0	0	0
	25	0.78	0	0	0
	50	0	0	0	0
	100	0.48	0	0.48	0

Table 4. Minimal resolution with temperature and BGE concentration. Capillary: 50µm i.d. x 57cm L_T x 360µm o.d.. Instrument conditions: 15kV (voltage), 200nm (wavelength), 10ºC (tray) and hydrodynamic injection 50mbar 5s. Sample: hsa-isomiR-16-5p + hsa-let-7g-5p + hsa-miR-21-5p in water at 5µM.

Separation of the 3 miRNAs (mixture 2) was successful under the previously mentioned conditions, but separation at baseline for all peaks could not be achieved (minimal $R_s < 1.5$).

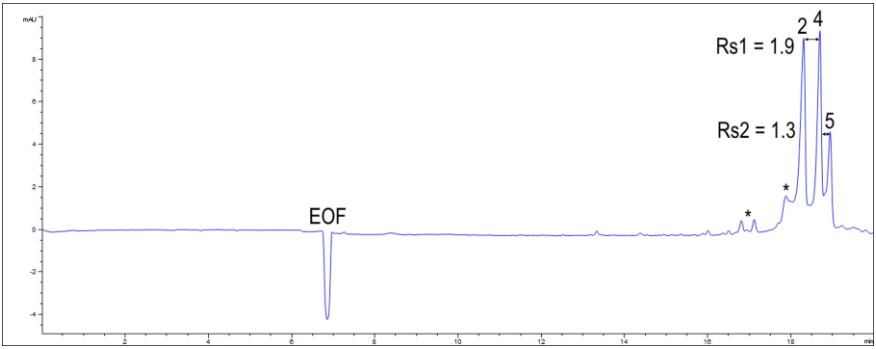


Figure 16. CE-UV electropherogram. BGE: NH4Ac 10mM pH9. Capillary: 50µm i.d. x 57cm L_T x 360µm o.d.. Instrument conditions: 15kV (voltage), 200nm (wavelength), 10ºC (cassette), 10ºC (tray) and hydrodynamic injection 50mbar 5s. Sample: hsa-isomiR-16-5p (5) + hsa-let-7g-5p (4) + hsa-miR-21-5p (2) in water at 5µM. R_{s1}=1.9 R_{s2}=1.3

6.1.3. CGE conditions

CGE was also tested during the course of this work, where PVA (89-98kDa) and PEG (7-9kDa) of different percentages were used as BGE additives. PEG at concentrations of 2.5, 7.5 and 12.5% (w/v) and PVA at 0.25, 0.5% and 1% (w/v) were tested. Reverse polarity mode (anode in the outlet) was used for separation due to EOF suppression. BGEs were centrifuged with Mikro 20 centrifuge with 2073 rotor (Hettich Zentrifugen, Tuttlingen, Germany) at 12600rpm for 10min. Conditioning steps were changed to 7min of BGE for preconditioning and 12min of water for postconditioning [27]. Detection of miRNA mixture with PEG was no possible, probably due to excessive viscosity of PEG solution. MiRNAs were detected with PVA until 0.5%, though no separation was observed (Figure 17). Capillary was eventually clogged at 1% PVA. Since no miRNA separation was achieved CGE was eventually discarded.

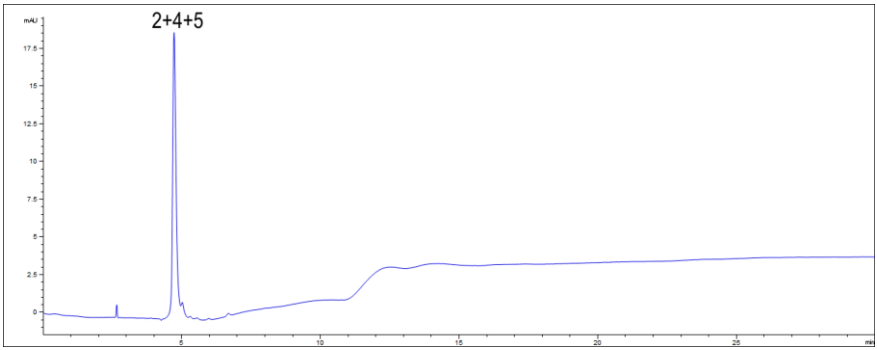


Figure 17. CE-UV electropherogram. BGE: NH4Ac 25mM pH9 + 0.5% PVA. Capillary: 75µm i.d. x 44cm x 360µm o.d.. Instrument conditions: -15kV (voltage), 200nm (wavelength), 30ºC (cassette), 25ºC (tray) and hydrodynamic injection 50mbar 10s. Sample: hsa-isomiR-16-5p (5) + hsa-let-7g-5p (4) + hsa-miR-21-5p (2) in water at 5µM.

6.1.4. CE-UV quality parameters

Several quality parameters of the optimized CE-UV method were evaluated:

- Repeatability: 10 replicates of the miRNA mixture (mixture 2) were analysed to test repeatability of the optimized method. Repeatability of migration times was overall good, with an RSD of 1.2% for EOF and an RSD 3 (2), 3.1 (4) and 3.1% (5) for each analyte. Peak height was chosen instead of peak area because of its higher repeatability (peak height went from 7 to 9% RSD while peak area went from 7 to 11% RSD).
- Linearity: Solutions with different concentrations of hsa-isomiR-16-5p were analysed for this part. Concentrations of 1, 5, 25, 50, 75 and 100µM were represented against peak height. Figure 18 shows this plot, along with its linear equation. This optimized method is linear from 1µM to 100µM (R^2=0.98).

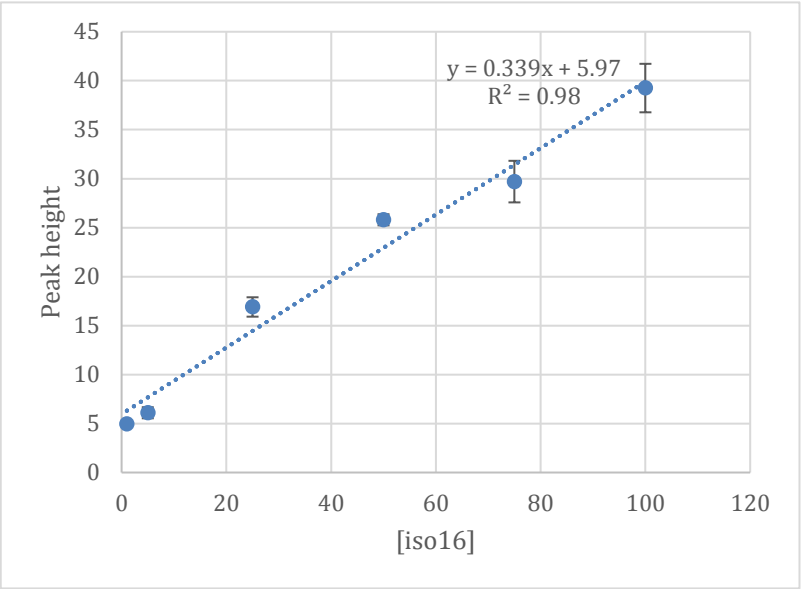


Figure 18. Plot of peak height vs. isomiR-16 concentration. The error bars stand for the standard deviation (n=3)

- Limit of detection (LOD): LOD was investigated for hsa-isomiR-16-5p. The experimental LOD was lower than 1µM (S/N = 4.9), as can be seen in Figure 19.

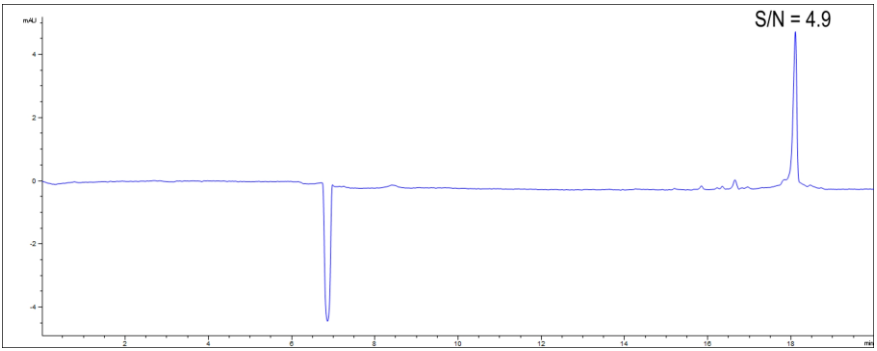


Figure 19. CE-UV electropherogram. BGE: NH4Ac 10mM pH9. Capillary: 50µm i.d. x 57cm L_T x 360µm o.d.. Instrument conditions: 15kV (voltage), 200nm (wavelength), 10ºC (cassette), 10ºC (tray) and hydrodynamic injection 50mbar 5s. Sample: hsa-isomiR-16-5p in water at 1µM. S/N=4.9.

6.2. OPTIMIZATION OF THE MALDI-TOF-MS METHOD

Instrumental parameters such as selection of the different acquisition and processing modes is discussed here, along with mass calibration and laser intensity. Usage of an internal standard as a calibrant (hsa-let-7g-5p) was studied due to the low accuracy of the linear mid mass negative mode. Theoretical mass used in the mass accuracy study was obtained from MicroRNA MultiTool software [28] and can be seen in Table 5. This calibration was done using a spot with only the internal standard inside. Laser intensity was optimized for the best S/N ratio. Finally, quality parameters of the optimized method were studied.

miRNA	+ Average [m/z]	+ Monoisotopic [m/z]	- Average [m/z]	- Monoisotopic [m/z]
hsa-miR-21-5p	7085.22	7081.90	7083.20	7079.89
hsa-let-7g-5p	7142.23	7138.89	7140.21	7136.87
hsa-isomiR-16-5p	7452.48	7448.99	7450.47	7446.98

Table 5. Reference mass obtained from MiRNA multitool software [23].

6.2.1. Instrumental parameters

- Laser intensity ranged from 400 to 7900 units. MiRNAs were detected starting from 6500 units. 6500, 7000 and 7500 units of laser intensity were tested. Intensities of 6500 yielded acceptable S/N ratios (S/N=300-500, Figure 20) without fragmentation of parent ion and were subsequently selected for MALDI-TOF-MS analysis. Under these conditions negative molecular ions with one charge (-1) were detected for the three miRNAs. Higher laser intensities were not used because S/N ratio didn't improve

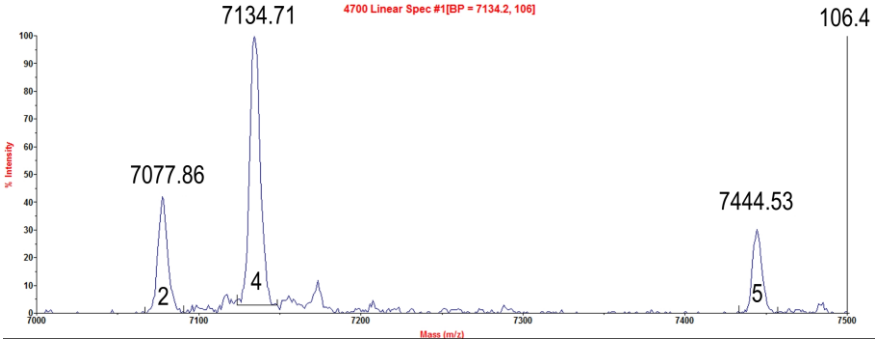


Figure 20. Mass spectra of MALDI-TOF-MS. Mode: linear mid mass negative. Laser intensity: 6500 units. Sample: hsa-miR-21-5p (2) + hsa-let-7g-5p (4) + hsa-isomiR-16-5p (5), spot prepared from 10µM mixture sample.

significantly.

- Mode selection: Linear mid mass and reflector for both positive and negative ions were tested for miRNA analysis. MS/MS mode could not be used due to instrument limitations with the mass range (maximum m/z=1500). Positive ion modes yielded lower S/N ratios than negative ones. Negative ion mode was selected as the best ionization mode. Reflector modes had the best mass accuracy out of all the modes (no calibration with an internal standard was required), though usage of 100μM concentrated miRNA solution was needed in order to detect the miRNAs (experimental LOD, Figure 21). This is because the mass range of the reflector modes goes as far as m/z=4000, meaning that the molecular ions with one charge (-1) could not be seen. The peaks detected were from the doubly charged ions (-2) of around 3500m/z. Isotopic distribution of peaks was also visible and is another factor for the bad LOD. Linear mid mass negative mode was the mode selected for miRNA characterization due to its far better experimental LOD, but no isotopic distribution could be resolved (Figures 20 and 22).

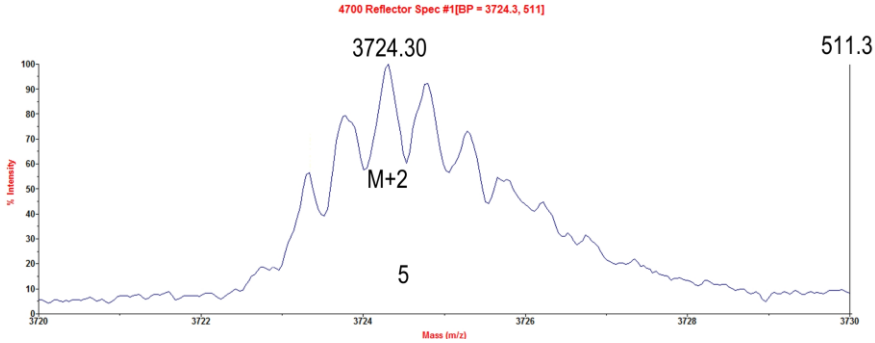


Figure 21. Mass spectra of MALDI-TOF-MS. Mode: reflector negative. Laser intensity: 6500 units. Sample: hsa-isomiR-16-5p (Peak molecular ion z=-2, M+2), spot prepared from 100μM concentrated sample.

- Calibration: Since linear mid mass negative mode presented some accuracy issues (mass error was approximately 0.15%) calibration with an internal standard was explored. Calibration using a miRNA (hsa-let-7g-5p) as an internal standard yielded mass errors of less than 0.02%, similar to those of reflector modes (less than 0.01%) and was therefore selected as part of the optimized method.

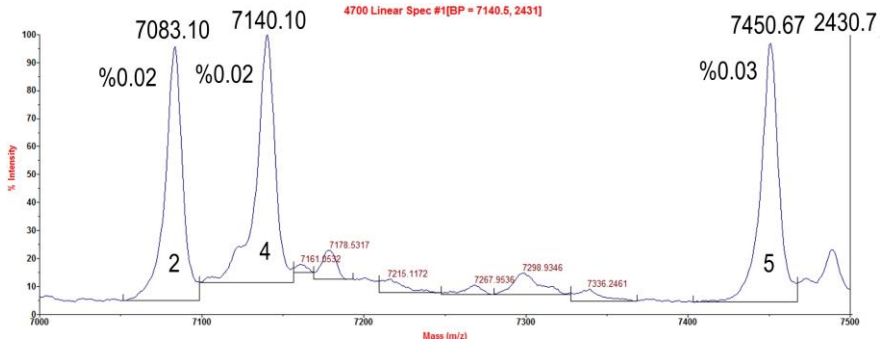


Figure 22. Mass spectra of MALDI-TOF-MS. Mode: linear mid mass negative. Laser intensity: 6500 units. Sample: hsa-miR-21-5p (2) + hsa-let-7g-5p (4) + hsa-isomiR-16-5p (5), spot prepared from 1µM mixture sample. Calibration: hsa-let-7g-5p (7140.21 reference). Mass accuracy is shown in sets ($|M_{theo}-M_{exp}|/M_{thep}$)*100.

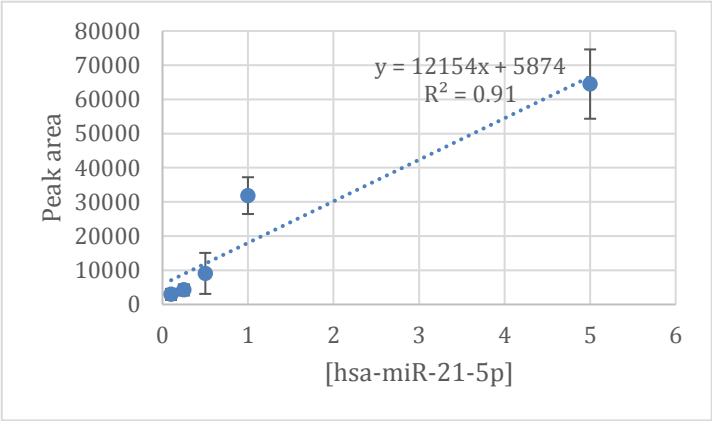
6.2.2. MALDI-TOF-MS quality parameters

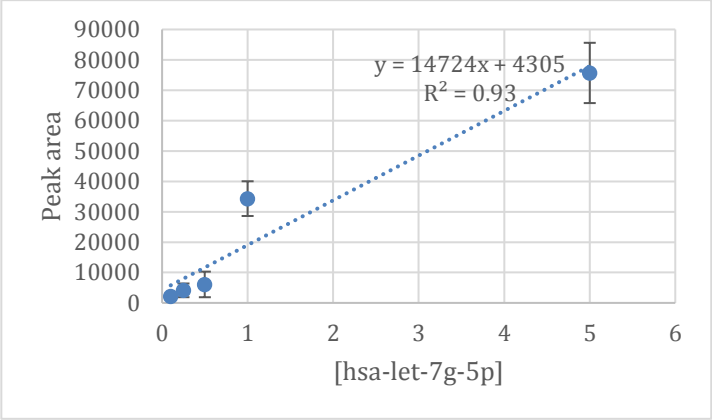
Several quality parameters of the optimized CE-UV method were evaluated:

- Mass accuracy: Using the MiRNA MultiTool software the theoretic average and monoisotopic molecular mass of the miRNAs were obtained [28]. A list of these masses can be found in Table 5. Mass differences for reflector modes were always less than 0.32m/z (mass accuracy was less than 0.01%). This is due to longer ion trajectories for reflector modes, allowing isotopic distribution to be resolved, as well as better mass accuracies. Linear mid mass negative without an internal standard yielded mass differences of more than 10m/z (mass accuracy was 0.15%). Post-transcriptional modifications of miRNAs may not be detected under those conditions, so calibration with an internal standard for miRNA characterization is mandatory with our method. The usage of hsa-let-7g-5p as an internal standard lead to mass differences of less than 1.4m/z (mass accuracy <0.02%).
- Repeatability: Three replicates of spots with concentrations of 0.1, 0.25, 0.5, 1 and 5µM were analysed for this part. Repeatability of mass was checked between replicates of the same concentration and between all other concentrations. Replicates for a given concentration gave an RSD of 0.002% and an RSD of 0.01% for all the other concentrations. Unfortunately, as expected in MALDI-TOF-MS peak height and peak area were not as repeatable, with values sometimes even arriving as far to 70%

RSD. This might be because of differences in matrix-analyte crystallization between spots.

- Linearity: Using the same conditions mentioned above linearity was checked in the concentration range between 0.1 and 5µM. Due to the low repeatability of peak height and area high standard deviations were observed. Figure 23-25 shows the plot between peak area and concentration for the different miRNAs, with error bars indicating the standard deviations. As expected, due to the low peak area repeatability, linearity was poor (R^2 ranged from 0.91 to 0.81). Therefore MALDI-TOF-MS was deemed unsuitable for quantitative analysis.





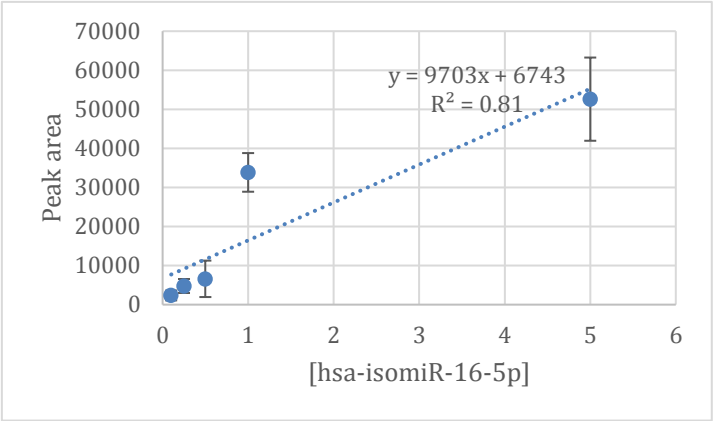


Figure 23-25. Plot peak area vs. miRNA concentration. Mode: linear mid mass negative. Laser intensity: 6500 units. Calibration: hsa-let-7g-5p (7140.21 reference). The error bars stand for the standard deviation (n=3)

- LOD: miRNAs were detected until 0.1µM (LOD), with S/N ratios of around 100. In Figure 26 a mass spectrum with the lowest concentration achieved is shown.

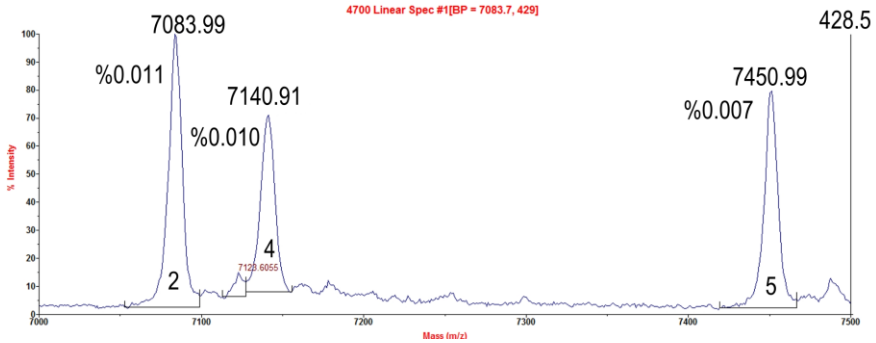


Figure 26. Mass spectra of MALDI-TOF-MS. Mode: linear mid mass negative. Laser intensity: 6500 units. Sample: hsa-miR-21-5p (2) + hsa-let-7g-5p (4) + hsa-isomiR-16-5p (5), spot prepared from 0.1µM mixture sample. Calibration: hsa-let-7g-5p (7140.21 reference). Mass accuracy is shown in sets ($|M_{theo}-M_{exp}|/M_{thep})*100$.

7. CONCLUSIONS

A simple and repeatable CE-UV method was optimized for separation of a mixture of three miRNAs (hsa-miR-21-5p, hsa-let-7g-5p and hsa-isomiR-16-5p), though complete baseline separation was not possible for all of them. A BGE of 10mM NH_4Ac at pH9 without additives was selected. The usage of additives (except cyclodextrin) did not improve separation. Sample was injected at 50mbar during 10s. Temperature was maintained at 10ºC for both the cassette and the sample tray and fused-silica capillaries of 50µm of i.d. with a total length of 57cm. The final optimized method is compatible with on-line MS detection, allowing in the future for CE-MS analysis. The next step from here should be increasing resolution, either through the usage of other and better BGEs or through the usage of additives that help in analyte separation. However, it is always challenging to make changes compatible with on-line MS detection.

MALDI-TOF-MS was also a pretty good characterization technique, being fast, highly accurate and having good LODs while also being simple, at least compared to the CE-UV method. Characterization of miRNAs was done in linear mid mass negative mode with a laser intensity of 6500 units. An internal standard (hsa-let-7g-5p) was used to correct mass accuracy issues. Unfortunately, sequencing studies using MALDI-TOF-MS could not be done due to instrument limitations, so sequencing of miRNAs by MS/MS was not possible.

Since our analytes were synthetic and purchased sample treatment was not needed, but study of miRNA from humans or animals will be much harder with these methods due to complexity of biological samples.

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9. ACRONYMS

MiRNA: MicroRNA

CE-UV: Capillary electrophoresis with ultraviolet-visible detection

LOD: Limits of detection

MALDI-TOF-MS: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

MS/MS: Tandem mass spectrometry

RNA: Ribonucleic acid

A: Adenine

C: Cytosine

G: Guanine

U: Uracil

Pri-miRNA: Primary microRNA

mRNA: MessengerRNA

RT-qPCR: Real time quantitative polymerase chain reaction

DNA: Deoxyribonucleic acid

LC: Liquid chromatography

CZE: Capillary zone electrophoresis

BGE: Background electrolyte

CE-MS: Capillary electrophoresis with on-line mass spectrometry detection

CGE: Capillary gel electrophoresis

q/r: Charge-to-ionic radius ratio

EOF: Electroosmotic flow

ESI: electrospray ionization

NGS: Next generation sequencing techniques

MEKC: Micellar electrokinetic chromatography

CMC: Critical micelle concentration

m/z: Mass-to-charge ratio

DAD: Photodiode array detector

Nd: YAG: Neodymium-doped yttrium aluminum garnet laser

NH4Ac: Ammonium acetate

NH4HCO3: Ammonium bicarbonate

H3BO3: Boric acid

Tris: Tris(hydroxymethyl)aminomethane

NaH2PO4 · H2O: Sodium dihydrogen phosphate monohydrate

MeOH: Methanol

ACN: Acetonitrile

HDMS-βCD: Heptakis-(2,3-dimethyl-6-sulfato)-β-cyclodextrin sodium salt

HFIP: 1,1,1,3,3,3-hexafluoro-2-propanol

TEA: Triethylamine

PFOA: Perfluorooctanoic acid

NH3: Ammonia

HCl: Hydrochloric acid

KOH: Potassium hydroxide

PVA: Poly (vinyl alcohol)

PEG: Polyethylene glycol

iPrOH: 2-propanol or isopropanol

THAP: 2',4',6'-Trihydroxyacetophenone monohydrate

AC: Ammonium citrate dibasic

i.d.: Internal diameter

L_T: Total capillary length

o.d.: Outer diameter

N_2: Nitrogen

R_s: Resolution

RSD: Relative standard deviation

S/N ratio: Signal/noise ratio.