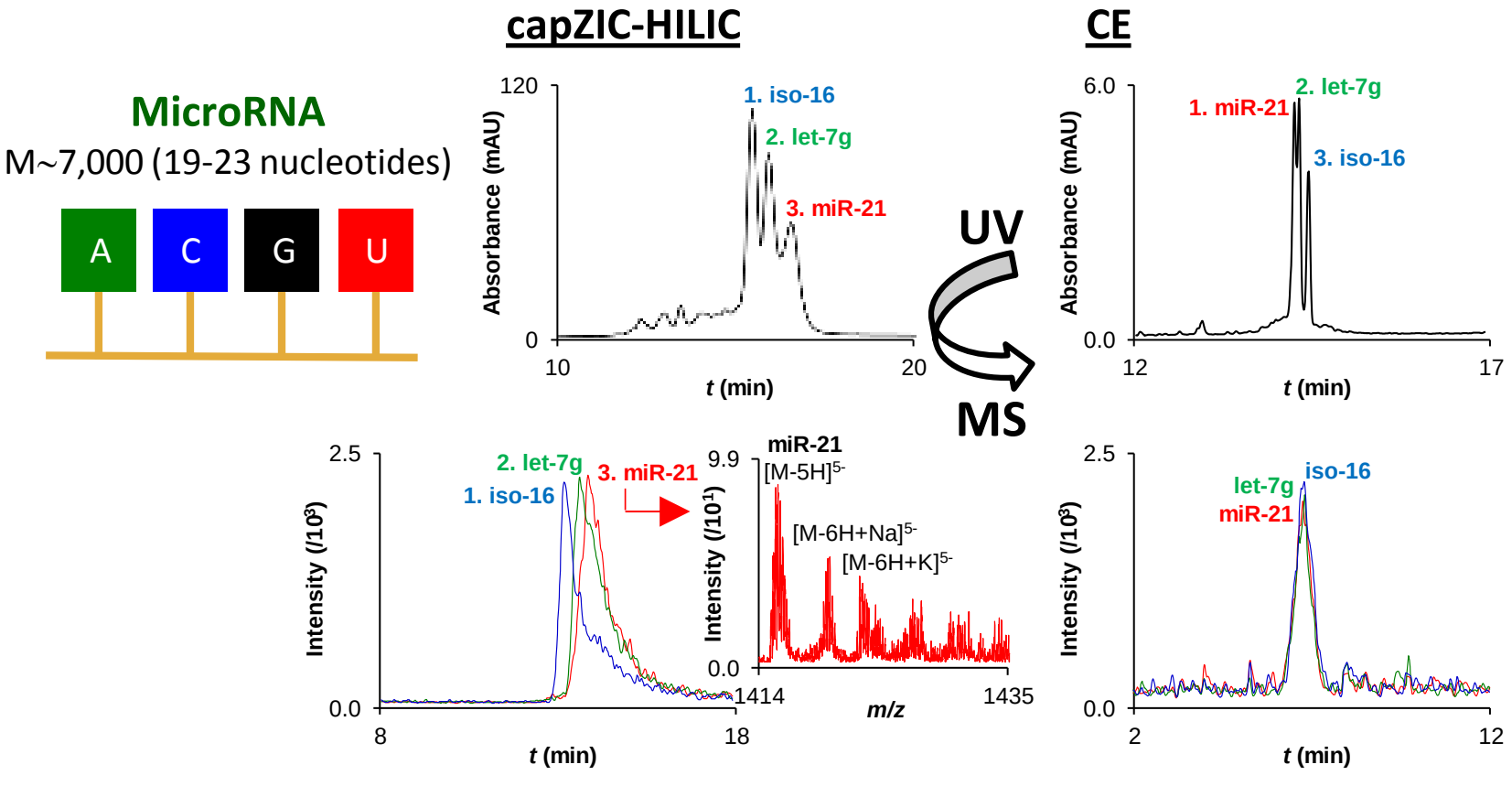
capZIC-HILIC
CE
MicroRNA
M~7,000 (19-23 nucleotides)
A
C
G
U
120
0
Absorbance (mAU)
10
20
t (min)
1. iso-16
2. let-7g
3. miR-21
UV
MS
6.0
0.0
Absorbance (mAU)
12
17
t (min)
1. miR-21
2. let-7g
3. iso-16
2.5
0.0
Intensity (/10³)
8
18
t (min)
2. let-7g
3. miR-21
1. iso-16
9.9
0.0
Intensity (/10¹)
1414
1435
m/z
miR-21
[M-5H]5-
[M-6H+Na]5-
[M-6H+K]5-
2.5
0.0
Intensity (/10³)
2
12
t (min)
let-7g
miR-21
iso-16

Highlights

- Direct, label-free and multiplex analysis of miRNAs is investigated.
- capZIC-HILIC and CZE with UV detection methods are optimized and validated.
- The established UV methods are transferred and validated with MS detection.
- The applicability to serum samples is evaluated.
- Methods comparison disclose their current pros and cons for miRNA analysis.

Comparison of capillary electrophoresis and zwitterionic-hydrophilic interaction capillary liquid chromatography with ultraviolet and mass spectrometry detection for the analysis of microRNA biomarkers

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KEYWORDS: Biomarker ; capillary electrophoresis ; capillary liquid chromatography ; mass spectrometry ; miRNA ; ZIC-HILIC

ABSTRACT

This study evaluates zwitterionic-hydrophilic interaction capillary liquid chromatography (capZIC-HILIC) and capillary electrophoresis (CE) with ultraviolet (UV) and mass spectrometry (MS) detection for the direct, label-free and multiplex analysis of microribonucleic acids (miRNAs). CapZIC-HILIC-UV and CE-UV methods were first optimized, resulting in similar separations for a mixture of three miRNAs (hsa-iso-miR-16-5p, hsa-let-7g-5p, and hsa-miR-21-5p) but with reversal of elution/migration orders and small differences in repeatability, linearity, limit of detection (LOD) and separation efficiency. The established UV methods were transferred and validated in these terms with mass spectrometry (MS) detection, which allowed identifying the miRNAs and characterizing their post-transcriptional modifications. LOD by capZIC-HILIC-MS was 1 µM of miRNA, around 5 times lower than by CE-MS due to the analyte dilution with the sheathflow CE-MS interface and to the slightly increased abundance of alkali metals adducts in the CE-MS mass spectra. In addition, the suction effect promoted by the nebulizer gas in CE-MS negatively affected the already compromised separations. In contrast, CE-MS showed superior repeatabilities with spiked serum samples, as well as reduced costs, extended capillary column durabilities and shorter conditioning times. The comparison of the different methods allows disclosing the current advantages and disadvantages of capZIC-HILIC and CE for the analysis of miRNA biomarkers.

Introduction

Small RNAs have attracted great interest in biomedical research because of their roles in cell cycle regulation to maintain a correct gene expression [1,2]. Microribonucleic acids (miRNAs) are short single stranded, non-protein-coding sequences of 19 to 23 ribonucleotides that have a crucial importance in processes like gene silencing and post-transcriptional regulation. miRNAs also act as signaling molecules that travel between different tissues through blood [2–4]. Several studies have associated miRNAs to different pathologies, especially cancer [2], hence they are regarded as potential biomarkers for clinical diagnosis [5,6].

Current routine methods for miRNA analysis allow indirect identification of the targeted miRNA sequences [7–9]. These methods include reverse transcription–quantitative polymerase chain reaction (RT-qPCR), which is the gold standard, next generation sequencing (NGS) and microarrays. However, these indirect methods show certain limitations. First, miRNAs must be retrotranscribed into complementary DNA before amplifying its concentration by PCR to enhance detection sensitivity [7–9]. In this process, the information related to post-transcriptional modifications of miRNAs is lost [10]. Another issue is that it is a targeted analysis, hence it is only possible to detect a miRNA if its primer is considered in the PCR mix [7–9]. In the case of entire RNA sequencing by NGS, the procedure needs additional steps like ligation adaptors and gene libraries creations that may produce mistakes or hamper the results [11].

The direct analysis of miRNA biomarkers is of great importance, but very complex due to their similar size and structure, as well as to their low concentration in biological samples. As miRNA are oligonucleotides, they are very polar compounds with ionizable phosphate groups. Therefore, capillary electrophoresis (CE) [12–17] and hydrophilic interaction capillary liquid chromatography (capHILIC) [18,19] can be

regarded nowadays as very suitable microscale separation techniques for their highly efficient separation and direct detection. Furthermore, under certain conditions these techniques are compatible with mass spectrometry (MS) detection [16–19], which allows identifying the separated miRNAs and characterizing their post-transcriptional modifications.

Capillary gel electrophoresis has been extensively applied to separate oligonucleotides, including miRNAs [12–15,20], but the typical sieving conditions are non-compatible with on-line MS detection. Separation conditions in capillary zone electrophoresis, hereafter referred to as CE, can be optimized for capillary electrophoresis-mass spectrometry (CE-MS). In CE, ionizable compounds as miRNAs can be separated according to their different charge-to-hydrodynamic radius ratios. Recently, the detection and characterization of miRNAs in human serum at concentrations down to 10 nM was demonstrated combining on-line preconcentration by sample stacking or solid-phase extraction with CE-MS [16,17]. However, none of these methods allowed separating the detected miRNAs and the identification solely relied on MS. Therefore, it is necessary to further expand our knowledge about the separation performance of CE for miRNA, as well as to explore other high-performance microscale separation techniques potentially compatible with on-line MS detection.

Nowadays, hydrophilic interaction liquid chromatography (HILIC) [18,19,21,22] is considered as a good alternative to CE [16,17] or reversed phase LC with ion-pairing reagents [22] for the separation of oligonucleotides. HILIC uses polar stationary phases with similar mobile phases to those in reversed-phase liquid chromatography. More recently, zwitterionic sulfoalkylbetaine and phosphorylcholine stationary phases have been introduced in the so-called zwitterionic HILIC (ZIC-HILIC). This particular type of HILIC stationary phase is recommended for the

separation of polar molecules with not enough electrical charge to use ion-exchange chromatography [23–26], like miRNAs. In this regard, moving from the dimensions of conventional LC into capillary LC (column diameters below 0.5 mm and flow rates of around 4 μL/min) is also desirable for the analysis of a minute volume of sample with miRNAs [27]. Nowadays, several authors have shown that HILIC and ZIC-HILIC can be used to analyze oligonucleotides with ultraviolet (UV) and MS detection [18,19,21,22]. However, to the best of our knowledge, the separation of miRNAs with capZIC-HILIC has not been yet demonstrated. In this study, CE and capZIC-HILIC with UV and MS detection are evaluated for the direct, label-free and multiplex analysis of three structurally related miRNAs (hsa-iso-miR-16-5p (iso-16), hsa-let-7g-5p (let-7g), and hsa-miR-21-5p (miR-21)). MiR-21 was the first serum miRNA biomarker discovered and it is a representative oncogenic miRNA [28]. Altered expression of miR-16 and let-7 families in cancer has also been observed [29]. The performance of the optimized methods is compared considering their repeatability, linearity, limit of detection, number of theoretical plates, separation resolution, conditioning time and column durability, as well as analyzing serum samples. The comparison intends to disclose the current advantages and disadvantages of capZIC-HILIC and CE for the analysis of miRNA biomarkers, as a starting point to further exploit their potential.

Experimental section

Materials and reagents

All solvents and reagents were analytical reagent grade or better. LC-MS quality acetonitrile (ACN), propan-2-ol, methanol and water were acquired from Panreac AppliChem (Barcelona, Spain). Ammonium bicarbonate, ammonium hydroxide (25%

m/m), boric acid, sodium dihydrogen phosphate and sodium hydroxide (≥99.0% m/m) were supplied by Merck (Darmstadt, Germany). Ammonium acetate (NH_4Ac, ≥99.999% m/m) was purchased from Sigma-Aldrich (Madrid, Spain). Synthetic miRNAs hsa-miR-16-5p with 3'-uridylation (iso-16), hsa-let-7g-5p (let-7g), and hsa-miR-21-5p (miR-21) were provided by Integrated DNA Technologies (Leuven, Belgium).

Preparation of miRNAs standards

The lyophilized miRNAs were resuspended in water to prepare 200 µM individual stock solutions, which were stored at -20 ºC until use. The stock solutions were diluted to 20 µM in water (CE) or ACN:water 50:50 (v/v) (capZIC-HILIC), filtered using a 0.22 µm polyvinyldene difluoride centrifugal filter (Ultrafree-MC, Millipore Bedford, MA, USA) at 11,000 x g for 4 min (25 ºC), and further diluted to the required working concentrations.

Preparation of serum samples

Blood was taken from a healthy volunteer, following standard operating procedures with the appropriate approval of the Ethical and Scientific Committees of the UB. Serum was prepared as described in our previous work [30]. Serum aliquots were stored in a freezer at -20 ºC when not in use. Serum samples were pretreated using a centrifugation-assisted solid-phase extraction kit (miRCURY™ RNA Isolation Kit, Exiqon, Hilden, Germany), which is recommended for purification and preconcentration of small RNAs [31]. Centrifugations and incubations (with moderate shaking) were done at 25 ºC. Two hundred µL of serum was centrifuged at 3,000 x g for 5 min and the supernatant was collected. Then, 60 µL of lysis solution was added. After vortexing and

incubating for 3 min, 20 µL of protein precipitation solution was added. The mixture was vortexed, incubated for 1 min and centrifuged at 11,000 x g for 3 min, before collecting the supernatant. Spiked serum samples were prepared adding at this point 2 µL of the 200 µM miRNAs stock solution. Then, 270 µL of propan-2-ol was added. The mixture was vortexed, transferred to a mini spin SPE column and incubated for 2 minutes. After centrifugation at 11,000 x g for 30 s, the mixture was successively washed with 100 µL of wash solution 1 (11,000 x g, 30 s), 700 µL of wash solution 2 (11,000 x g, 30 s) and 250 µL of wash solution 2 (11,000 x g, 2 min). The mini spin SPE column was placed in a new collection tube and retained miRNAs were finally eluted with 50 µL of water centrifuging at 11,000 x g for 1 min. Blank and spiked serum samples were analyzed by CE-MS and, after dilution 1:1 (v/v) (sample:ACN), by capZIC-HILIC-MS.

Apparatus

pH measurements were made with a Crison 2002 potentiometer and 52-03 electrode (Crison Instruments, Barcelona, Spain). Incubations were performed in a TS-100 thermoshaker (Biosan, Riga, Latvian Republic). Centrifugal filtration was carried out at 25 ºC in a cooled Rotanta 460 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany).

Instrumentation

CapZIC-HILIC experiments were performed in a Dionex Ultimate 3000 RSLCnano chromatograph (Thermo Fisher Scientific, Massachusetts, USA) with a UV-absorption spectrophotometric diode array detector (UV-DAD). CapZIC-cHILIC columns (150 x 0.3 mm, 3 µm and 100Å particles with phosphorylcholine) were

purchased from Merck. Chromeleon™ chromatography data System software (Thermo Fisher Scientific) was used for capLC control, UV data acquisition and processing.

CE experiments were performed in a 7100 series capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany) with a UV-DAD. Fused silica capillaries were supplied by Polymicro Technologies (Phoenix, AZ, USA). ChemStation software (Agilent Technologies) was used for CE control, UV data acquisition and processing.

The UV data was acquired at a wavelength of 260 nm. CapZIC-HILIC-MS and CE-MS experiments were performed with a 6220 time-of-flight (TOF) mass spectrometer and an orthogonal electrospray ionization (ESI) interface (Agilent Technologies). A microsprayer and a co-axial sheathflow interface were used for capLC-MS and CE-MS, respectively. MS operation and data acquisition were done using MassHunter Workstation software (Agilent Technologies). Qualitative Analysis software was used for data analysis.

Zwitterionic-hydrophilic interaction capillary liquid chromatography

The optimized mobile phase consisted in 5 mM NH_4Ac (pH 6.8, without pH adjustment) (A) and ACN (B). Both solvents were filtered through 0.2 µm nylon filters (Macherey-Nagel, Düren, Germany), degassed by ultrasonication for 10 min just before use and replaced every three days. The optimized separations were performed at 30 ºC and consisted in a linear gradient at a flow rate of 4 µL/min (0-5 min, 70-60% v/v ACN; 5-30 min, 60-20% v/v ACN; 30-35 min, 20-5% v/v ACN; 35-50 min, 5-5% v/v ACN; 50-55 min, 5-70% v/v ACN; 55-65 min, 70-70% v/v ACN).

In order to obtain the best repeatability, it was necessary to equilibrate the column for 2 hours under the initial gradient conditions before a sequence of analyses.

A blank analysis was done before analyzing a miRNA solution or spiked serum sample of a certain concentration. Analyses at each concentration level were repeated four times and the first chromatogram after the blank analysis was always discarded due to poor repeatability. Two hundred nL of sample was injected using the microliter pick up mode (i.e., the injected sample was placed between two plugs of starting mobile phase until completing the injection loop volume (20 µL)).

For capZIC-HILIC-MS, the optimized MS parameters in negative ESI mode were: capillary voltage 3500 V, drying gas temperature 350 °C, drying gas flow rate 6 L/min, nebulizer gas pressure 10 psig (69 kPa), fragmentor voltage 225 V, skimmer voltage 70 V, octopole frequency voltage 300 V. Data were collected in profile at 1 spectrum/s between 100 and 3,200 *m/z*, with the mass range set to high resolution mode (4 GHz).

Capillary electrophoresis (CE)

CE-UV separations were performed at 10 °C in a 57 cm length (L_T) × 50 µm i.d. × 365 µm o.d. fused silica capillary. The UV window was placed at 48.5 cm from the inlet of the capillary. The optimized background electrolyte (BGE) was 10 mM NH_4Ac adjusted to pH 9.0 with ammonium hydroxide. All capillary rinses were performed at high pressure (930 mbar). New capillaries, and between workdays, were activated by flushing with water (10 min), 1 M NaOH (10 min), water (10 min) and BGE (10 min). The samples were hydrodynamically injected at 50 mbar for 5 s (8 nL, i.e., 0.7% of the capillary, estimated using the Hagen–Poiseuille equation [32]), and a separation voltage of +20 kV was applied (normal polarity, cathode in the outlet). The autosampler was kept at 10 °C using an external water bath (Minichiller 300, Peter Huber

196 Kältemaschinenbau AG, Offenburg, Germany). Between consecutive runs, the capillary
197 was conditioned by flushing with water (2 min) and BGE (5 min).

198 For CE-MS analysis, a fused silica capillary of 72 cm L_T × 75 µm i.d. × 365 µm
199 o.d. was used. The optimized BGE consisted in 25 mM NH_4Ac (pH 6.8, without pH
200 adjustment). The sheath liquid was propan-2-ol:water 80:20 (v/v) and was delivered at
201 3.3 µL/min by a KD Scientific 100 series infusion pump (Holliston, MA). The BGEs
202 and sheath liquid were ultrasonicated for 5 min and filtered through 0.2 µm filters
203 before use. The activation of the capillary was performed off-line to avoid the
204 unnecessary contamination of the MS instrument. The samples were hydrodynamically
205 injected at 50 mbar for 10 s (60 nL, i.e., 1.9% of the capillary [32]), and a separation
206 voltage of +20 kV was applied (normal polarity, cathode in the outlet). The optimized
207 MS parameters were the same as for capZIC-HILIC-MS except for the nebulizer gas
208 pressure (7 psig (48 kPa)).

209

210 **Quality parameters**

211 Linearity ranges were studied analyzing in triplicate mixtures of iso-16 and miR-
212 21 between 0.2 and 20 µM. An estimation of the LODs was obtained by analyzing
213 standard mixtures at low concentration (close to the LOD level defined for a signal-to-
214 noise ratio (S/N) of 3, n=3).

215 Repeatability studies were carried out by analyzing a mixture of 10 µM of iso-16
216 and miR-21 miRNAs (n=3) and the relative standard deviation (%RSD) of peak areas
217 and retention or migration times (t_r or t_m) were calculated. The separation performance
218 for iso-16 and miR-21 was evaluated by calculating the resolution ($R_s=1.18*(t_2-$
219 $t_1)/(w_{2,1/2}+w_{1,1/2})$) and the number of theoretical plates ($N=5.54*(t/w_{1/2})^2$), where t and
220 $w_{1/2}$ are the t_r or t_m and peak width at half height for the miRNAs.

The relative molecular mass (M_r) of the miRNAs was calculated from the monoisotopic mass-to-charge ratio (m/z) of the most abundant molecular ion ($[M-5H]^{5-}$, **Table 1**). Extracted ion chromatograms (EICs) and extracted ion electropherograms (EIEs) were obtained considering the m/z values of the first six peaks of the isotopic distribution for $[M-5H]^{5-}$ (e.g., for miR-21, m/z range from 1415.17 to 1416.17).

Results and discussion

Three synthetic miRNAs (iso-16, let-7g, and miR-21) with slight structural differences were used for the optimization of capZIC-HILIC and CE methods with UV and MS detection. The sequence, M_r and modifications of the three miRNAs are presented in **Table 1.**

Analysis of miRNAs by capZIC-HILIC

A capZIC-HILIC-UV method was optimized by analyzing 20 µM standard solutions of the three synthetic miRNAs. HILIC presents three different mechanisms to explain analyte separation, namely hydrogen bonding, electrostatic interactions and hydrophilic partitioning [23,24,26]. The last one is the most widely accepted. Therefore, changes in water composition can significantly affect column performance because it may cause variations in the water layer thickness inside the column [33]. In order to avoid poor analysis reproducibility, extensive washing and column re-equilibration steps were included in all the studied separation conditions. The chromatograms for the tested conditions are presented in **Figure S-1**. Although, the three miRNA could not be baseline separated, three peaks were clearly detected under the final selected conditions, namely sample solvent with 50% v/v ACN (**Figure S-1A(ii)**), 5 mM NH_4Ac and pH 6.8 as aqueous mobile phase (**Figure S-1B(ii) and S-1C(ii)**, respectively), and column

temperature at 30 °C (**Figure S-1D(i)**). Several gradient rates were also investigated to improve separation efficiency and resolution (**Figure S-1E**). Gradient 1 started at 70% v/v ACN and linearly decreased to 1% v/v ACN during 35 min (2.0% v/v ACN decrease/min). Gradient 2 started at 70% v/v ACN, linearly decreased to 60% v/v ACN during 5 min and, then, linearly decreased to 20% v/v ACN in 25 min (1.6% v/v ACN decrease/min). Gradient 3 started at 60% v/v ACN and linearly decreased to 40% v/v ACN during 35 min (0.6% v/v ACN decrease/min). The best compromise between peak shape, separation efficiency and resolution and total analysis time was obtained with gradient 2 (**Figure S-1E (ii)**), which was selected for the rest of experiments.

Figure 1A shows the separation of a 20 µM mixture of the three miRNAs by capZIC-HILIC-UV under the optimized conditions. Iso-16 (the less hydrophilic) was detected first at 15.6 min, then let-7g at 16.0 min, and miR-21 (the most hydrophilic) at 16.7 min. The optimized capZIC-HILIC-UV method was then evaluated with MS detection. **Figure 1B(i)** shows the EICs of a 5 µM mixture of the studied miRNAs by capZIC-HILIC-MS. The three miRNAs were again slightly separated but could be easily identified and resolved considering their different M_r. They eluted a little earlier due to the shorter post-column path, at 13.3 min (iso-16), 13.7 min (let-7g) and 14.0 min (miR-21). Peak broadening and lower peak separation were observed, probably due to the internal diameter of the metal tube used as electrode in the capLC-MS microsprayer (e.g., electrode internal diameter).

As an example of the mass spectra obtained for the miRNAs by capZIC-HILIC-MS, **Figure 1B(ii)** shows the mass spectrum for miR-21. The most abundant ion was the $[M-5H]^{5-}$, and some Na^+ and K^+ adducts were detected ($[M-6H+Na]^{5-}$ and $[M-6H+K]^{5-}$). The presence of alkali metal adduct ions splits the signal for the $[M-5H]^{5-}$ ions, hindering the interpretation of the mass spectra and overall decreasing the

sensitivity [17]. **Table 1** shows the calculated M_r for the studied miRNAs, including their post-transcriptional modifications, and mass accuracy was good in all cases (error < 10 ppm).

With regard to the lifetime of the capZIC-cHILIC columns, three different columns were tested and they performed well an average lifetime of 150 injections before column bleeding was detected by MS. This affected peak shape, resolution and t_r. This limited durability of HILIC columns has been also reported by other authors [24,34].

Analysis of miRNAs by CE

A CE-UV method for the separation of miRNAs was optimized by evaluating the BGE composition (pH and ionic strength), the inner diameter of the fused silica capillary, the injection time and pressure, the separation temperature and the voltage (**Figure S-2**). BGEs with a calculated ionic strength of approximately 25 mM were tested (i.e., 25 mM NH_4Ac, 20 mM NH_4HCO_3, 60 mM H_3BO_3 and 10 mM NaH_2PO_4 at pH 8.0, 9.0 and 10.0). The best results were achieved with a BGE of 25 mM NH_4Ac, pH 9.0. Separations were tested in fused silica capillaries of 50 and 75 µm i.d., obtaining better resolutions with the narrower capillary (**Figure S-2A(i)**). The best compromise between separation resolution, sensitivity and total analysis time was observed injecting the sample hydrodynamically for 5 s at 50 mbar (**Figure S-2B(iii)**) and separating at 20 kV (**Figure S-2C(iii)**). Preliminary experiments showed some interaction between the effect of ionic strength and temperature. Therefore, BGEs of NH_4Ac at concentrations from 10 to 100 mM and separation temperatures from 10 to 55 ºC were tested in a 4^2 factorial experimental design. As can be seen in the graph of **Figure S-2D** and the

295 electropherogram of **Figure S-2E(iv)**, the best resolution was achieved with 10 mM
296 NH_4Ac, pH 9.0, at 10 ºC.

297 **Figure 2A** shows the separation of a 5 µM mixture of the three miRNAs by CE-
298 UV under the optimized conditions. The BGE was always freshly prepared because
299 aging negatively affected miRNAs separation. As can be observed, the miRNAs were
300 not again baseline resolved and total separation times were similar compared to capZIC-
301 HILIC-UV (around 15 minutes). However, CE-UV did not require the long washing
302 and re-equilibration steps of capZIC-HILIC-UV. Moreover, CE-UV uses no organic
303 solvents and fused silica capillaries are cheap and can be easily replaced and activated.
304 Regarding the migration order of the miRNAs by CE-UV (**Figure 2A**), it was reversed
305 compared to capZIC-HILIC-UV (**Figure 1A**), indicating an inverse correlation between
306 the charge-to-radius ratio and hydrophilic partitioning mechanisms governing
307 separation in both techniques.

308 The optimized CE-UV method was evaluated with MS detection, and the
309 already compromised CE-UV separations were completely lost by CE-MS. This was
310 probably due to the impossibility to thermostatize to 10 ºC the segment of separation
311 capillary located outside of the CE cartridge cassette, the suction effect promoted by the
312 nebulizer gas and the analyte dilution inherent to the sheathflow interface [35]. Lower
313 nebulizer gas pressures and sheath liquid flow rates than 7 psig (48 kPa) and 3.3
314 µL/min, respectively, did not produce a reproducible spray, making it impossible to
315 further increase separation resolution. Then, the i.d. of the separation capillary was
316 increased from 50 to 75 µm to at least counteract analyte dilution injecting a larger
317 volume of sample (10 s at 50 mbar were 60 nL in a 75 µm i.d. capillary vs 12 nL in a 50
318 µm i.d. capillary [32]). Furthermore, the concentration and pH of the BGE optimized for
319 CE-UV (i.e., 10 mM NH_4Ac pH 9.0) was changed to 25 mM NH_4Ac, pH 6.8, in order

to lower the presence of alkali metal adducts and hence, increasing sensitivity. **Figure 2B(i)** and **2B(ii)** show the EIEs of a 5 µM mixture of the studied miRNAs by CE-MS and the mass spectrum for miR-21, respectively. Compared to capZIC-HILIC-MS (**Figure 1B(ii)**), the mass spectrum of **Figure 2 B(ii)** exhibited the same ion clusters but a slight increase in Na^+ and K^+ adducts was detected. The higher abundance of the alkali metal adducts was probably due to the use of a bare fused silica capillary for the separation, which is typically activated flushing with NaOH.

Comparison of quality parameters

The developed capZIC-HILIC-UV, capZIC-HILIC-MS, CE-UV and CE-MS methods were validated with standard mixtures of iso-16 and miR-21 (i.e., the pair of miRNAs that showed the best separation resolution with UV detection).

Table 2 shows the quality parameters of the established methods. Linearity of peak area versus concentration was investigated in the concentration range between 0.2 µM and 20 µM. CE-UV presented the widest linear range (1–20 µM). Linearity range by capZIC-HILIC-UV and CE-MS was slightly shorter (5-20 µM), but not as short as by capZIC-HILIC-MS (1-10 µM).

Regarding the LODs, capZIC-HILIC-UV and capZIC-HILIC-MS showed a similar performance to CE-UV (1 µM, **Table 2**). In CE-MS, the LODs were slightly higher (5 µM, **Table 2**), probably due to analyte dilution promoted by the sheath liquid in the sheathflow CE-MS interface and to the slightly increased abundance of alkali metals adducts in the CE-MS mass spectra. In terms of repeatability, adequate results were obtained with the four methods. %RSD (n=3) were lower than 7.5% for peak areas and lower than 4.1% for t_r or t_m. As expected, the largest %RSD in peak areas were

obtained with MS detection and repeatabilities in t_m by CE were lower than in t_r by capZIC-HILIC.

The separation performance with the different methods was evaluated in terms of separation efficiency and resolution, calculating N and R_s (**Table 3**). The best separation efficiency was obtained by CE-UV, presenting N values in the 10^5 range. Separation efficiency was slightly higher by capZIC-HILIC-UV than by capZIC-HILIC-MS and CE-MS. Regarding R_s, values of around 1.4 were obtained by capZIC-HILIC-UV and CE-UV, but decreased until 0.44 by capZIC-HILIC-MS and the miRNAs totally comigrated by CE-MS. In order to further increase peak resolution, it would be required to explore the use of MS compatible additives in the BGE or the mobile phase, as well as coated capillaries or novel HILIC stationary phases. In CE-MS, it would be also interesting to investigate the separation performance with sheathless CE-MS interfaces. Anyway, improving separation resolution between miRNA is an extremely challenging task due to their structural similarity.

Analysis of serum samples

The applicability of the capZIC-HILIC-MS and CE-MS methods for the analysis of biological samples was evaluated with human serum samples spiked with iso-16 and miR-21 at 2 µM. Serum samples were pretreated before the analysis using a commercially available kit for off-line purification and preconcentration of small RNAs. In terms of t_r, similar results were obtained for serum samples and standards by capZIC-HILIC-MS (**Figures 3A(i)** and **1B(i)**). However, the separation resolution between the miRNAs in serum samples was slightly lower than for the analysis of standards (compare R_s values in **Table 3**) mainly due to the worse separation efficiency (N values were 3-4 times lower and %RSD values for N were slightly higher in serum samples,

Table 3). This was probably due to the remaining serum matrix components. Comparing the mass spectra of the miRNAs for serum samples and standards (**Figures 3A(ii)** and **1B(ii)**), an increase in Na^+ adducts was detected in serum samples. Regarding CE-MS, the analysis of the spiked serum samples gave similar results compared to standards in terms of t_m and separation efficiency, and again both miRNA comigrated (see **Figures 3B(i)** and **2B(i),** and **Table 3**). As in capZIC-HILIC-MS, an increase of Na^+ adducts was also detected in the mass spectrum (compare **Figures 3B(ii)** and **2B(ii)**).

As expected, no endogenous miRNAs were detected in non-spiked serum samples by capZIC-HILIC-MS and CE-MS, because the concentration of these low abundant biomarkers in healthy controls is far lower than the current LODs. Therefore, preconcentration of the miRNAs and sensitive mass spectrometers are required to expand the applicability of MS in this field, but some advancements are being made in this direction. As recently showed, combining on-line preconcentration by sample stacking or solid-phase extraction with CE-MS for detection of circulating miRNAs in serum samples of patients with advanced chronic lymphocytic leukemia [16,17]. However, none of these methods allowed separating the detected miRNAs and the identification solely relied on MS. Therefore, it is necessary to further expand our knowledge about miRNAs separation.

Concluding remarks

CapZIC-HILIC-UV, capZIC-HILIC-MS, CE-UV, and CE-MS methods for the separation, direct detection and characterization of miRNAs were optimized and validated. Similar figures of merit were obtained by capZIC-HILIC-UV and CE-UV in terms of linearity range, LOD and separation resolution, but higher separation efficiency

was obtained by CE-UV. Interestingly, reversal of elution/migration orders was observed, suggesting an inverse correlation between separation selectivities of both techniques. The established UV methods were transferred and validated with MS detection. LODs by capZIC-HILIC-MS were slightly lower than by CE-MS (i.e., around 5 times). Furthermore, a smaller amount of alkali metal adduct formation was observed and the poor separations between miRNAs were preserved to a large extent. In contrast, CE-MS showed superior repeatabilities with spiked serum samples, in addition to reduced costs, extended capillary column durability and shorter conditioning times. CE and capZIC-HILIC show a great potential for the direct, label-free and multiplex analysis of miRNAs in biological fluids, but together with the limited sensitivity, this study evidences that further improvements must be made in separation resolution. This fair and detailed comparison between CE and capZIC-HILIC settles the starting point to progress regarding this issue. Conclusions drawn for the analysis of miRNA can be also extended to other small oligonucleotides, such as novel biopharmaceuticals.

Supporting Information

Optimization of capZIC-HILIC-UV and CE-UV methods.

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The authors declare no conflicts of interest.

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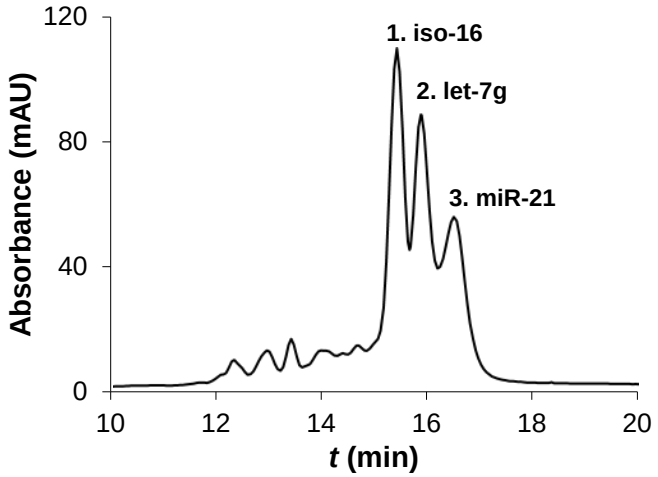
Figure captions

Figure 1. Separation of a standard mixture of iso-16, let-7g, and miR-21 by capZIC-HILIC with A) UV detection (20 µM) and B) MS detection (5 µM): i) extracted ion chromatograms (EICs), and ii) miR-21 mass spectrum.

Figure 2. Separation of a 5 µM standard mixture of iso-16, let-7g, and miR-21 by A) CE-UV and B) CE-MS: i) extracted ion electropherograms (EIEs), and ii) miR-21 mass spectrum.

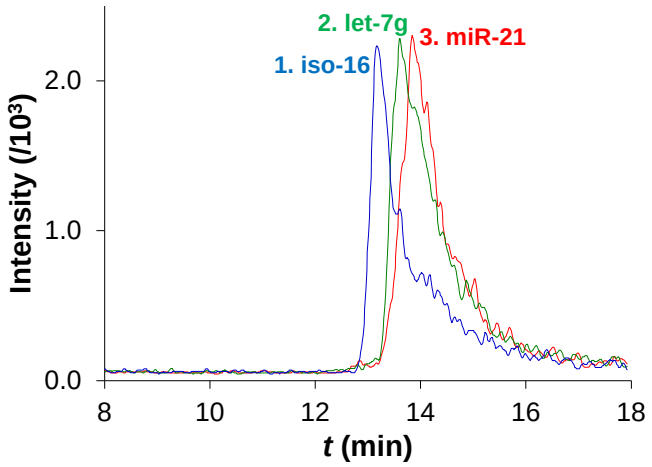
Figure 3. Analysis of a serum sample spiked with 2 µM of iso-16 and miR-21 and pretreated by centrifugation-assisted solid-phase extraction before A) capZIC-HILIC-MS and B) CE-MS: i) extracted ion chromatograms or electropherograms (EICs or EIEs), and ii) miR-21 mass spectrum.

A) capZIC-HILIC-UV



B) capZIC-HILIC-MS

i) EICs



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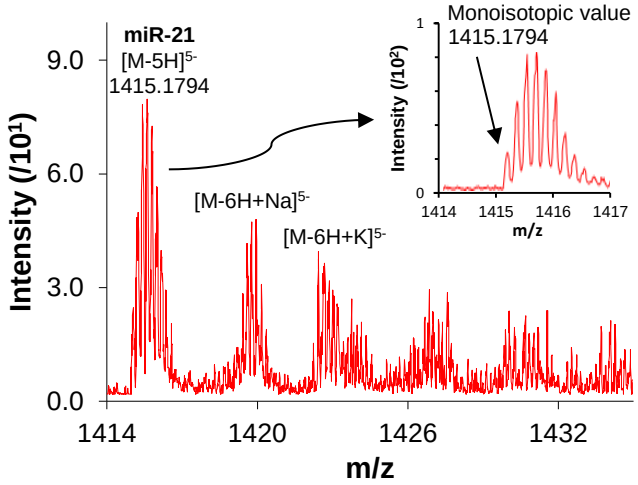
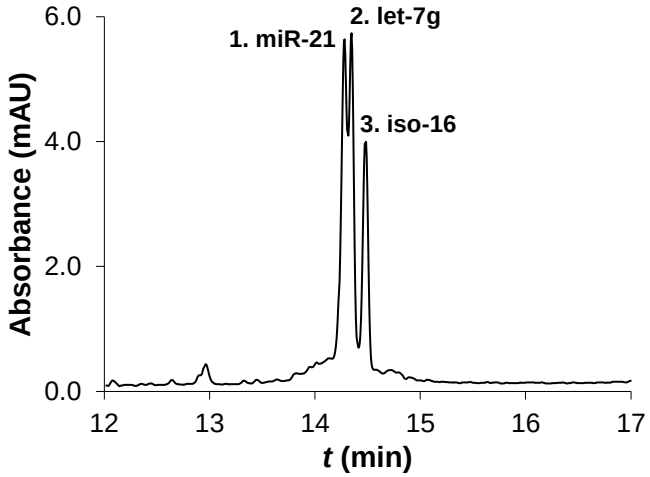


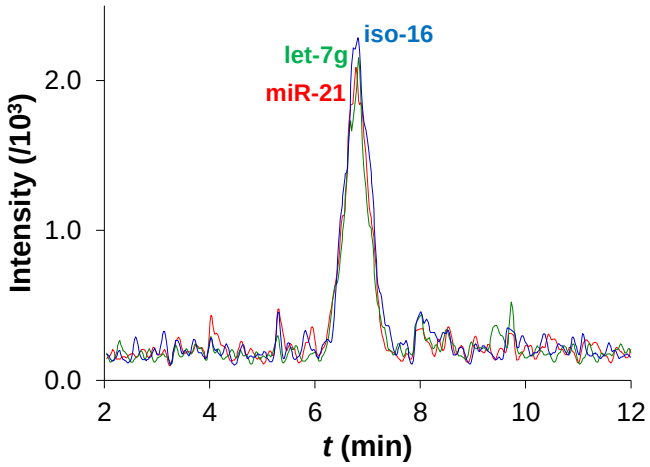
Figure 1

A) CE-UV



B) CE-MS

i) EIEs



ii) MS

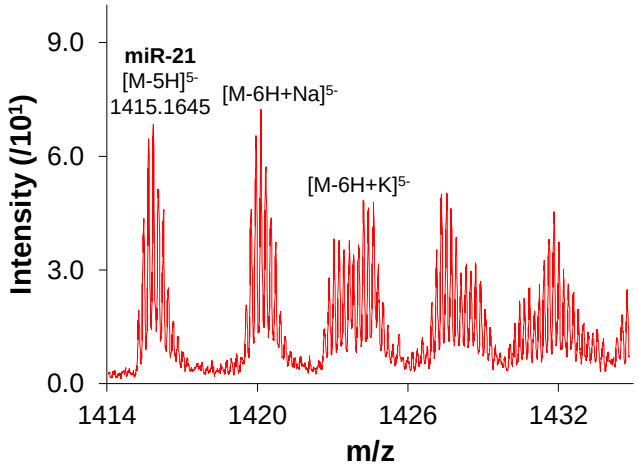


Figure 2

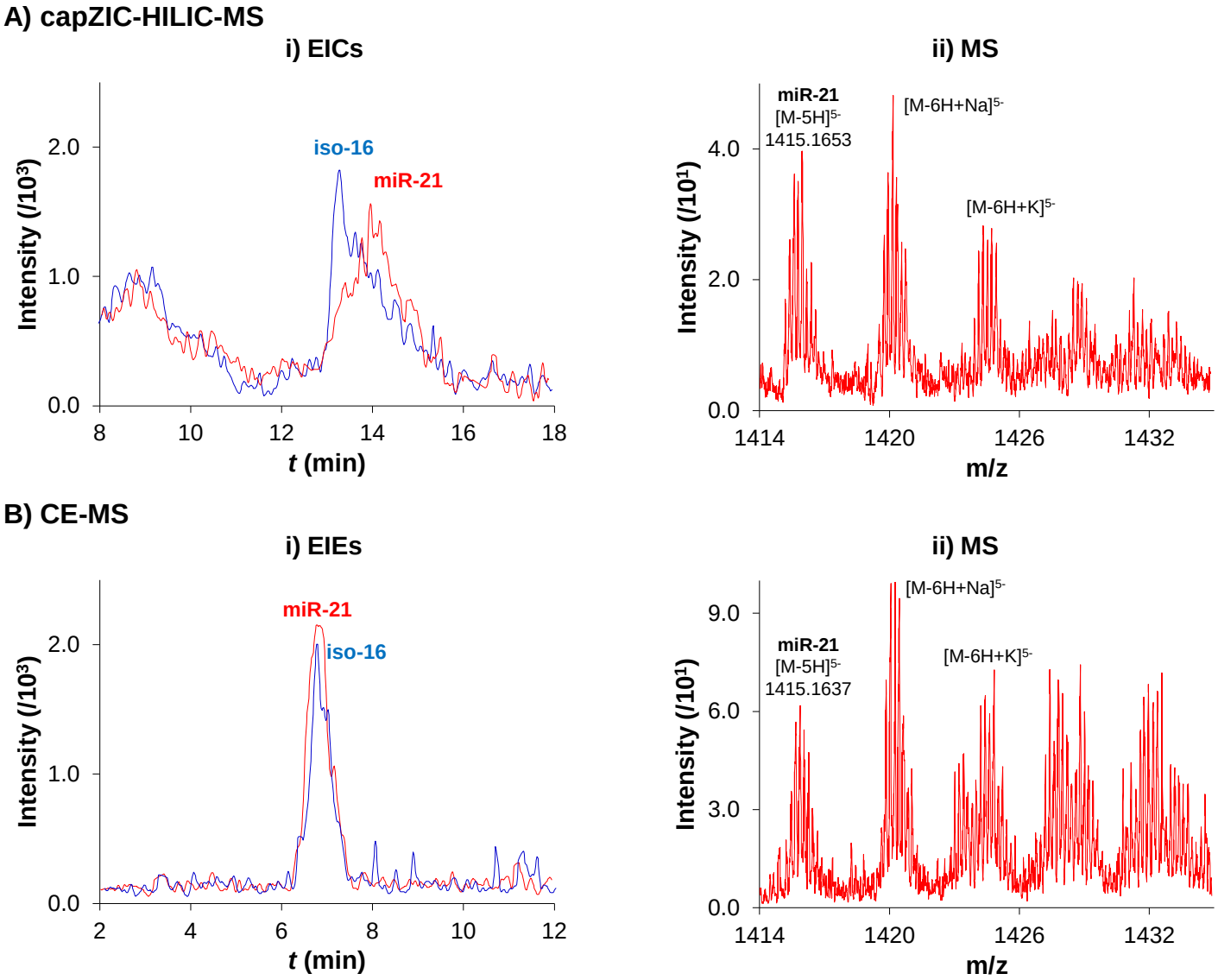

A) capZIC-HILIC-MS
i) EICs
iso-16
miR-21
Intensity (/10³)
2.0
1.0
0.0
8
10
12
14
16
18
t (min)
ii) MS
miR-21
[M-5H]⁵⁻
1415.1653
[M-6H+Na]⁵⁻
[M-6H+K]⁵⁻
Intensity (/10¹)
4.0
2.0
0.0
1414
1420
1426
1432
m/z
B) CE-MS
i) EIEs
miR-21
iso-16
Intensity (/10³)
2.0
1.0
0.0
2
4
6
8
10
12
t (min)
ii) MS
[M-6H+Na]⁵⁻
miR-21
[M-5H]⁵⁻
1415.1637
[M-6H+K]⁵⁻
Intensity (/10¹)
9.0
6.0
3.0
0.0
1414
1420
1426
1432
m/z

Figure 3

Table 1. Characteristics and relative molecular mass (M_r) of the standard miRNAs.

miRNA ID	Length, nt	Sequence	Modifications	m/z [M-5H]$^{5-}$ [a]		Calculated M_r [b]		
				Theoretical	Experimental	Theoretical	Experimental	Error (ppm) [c]
iso-miR-16-5p (iso-16)	23	5’ UAGCAGCACGUAAAUAUUGGCGU 3’	5’ phosphorylation and 3’ uridylation	1488.5890	1488.6018	7447.98	7448.02	9
hsa-let-7g-5p (let-7g)	22	5’ UGAGGUAGUAGUUUGUACAGUU 3’	5’ phosphorylation	1426.5677	1426.5701	7137.88	7137.89	1
hsa-miR-21-5p (miR-21)	22	5’ UAGCUUAUCAGACUGAUGUUGA 3’	5’ phosphorylation	1415.1707	1415.1794	7080.89	7080.94	7

[a] Experimental values were obtained by capZIC-HILIC-MS.

[b] M_r was calculated as monoisotopic mass.

[c] The relative error was calculated in ppm as: (M_r exp – M_r theo)/M_r theo × 10^6 (exp = experimental and theo = theoretical). M_r exp was obtained as an average of three replicates.

Table 2. Linear regression equation, linearity range, limit of detection and repeatability for the analysis of iso-16 and miR-21 by capZIC-HILIC-UV, capZIC-HILIC-MS, CE-UV and CE-MS.

miRNA	Method	Linearity		LOD (µM) (S/N>3) [a]	Repeatability, %RSD (n=3, 10 µM)	
		A = b*C + a, (R^2>0.99)	Range (µM)		Peak area	t_r or t_m
iso-16	CapZIC-HILIC-UV	A= 1.94 C + 5.85	5 - 20	1.0	3.5	0.76
	CapZIC-HILIC-MS	A= 18994 C + 6493	1 - 10	1.0	5.9	0.19
	CE-UV	A= 3.26 C + 0.71	1 - 20	1.0	3.8	1.2
	CE-MS	A= 2520 C - 8388	5 - 20	5.0	3.4	3.7
miR-21	CapZIC-HILIC-UV	A= 1.76 C + 2.25	5 - 20	1.0	3.6	0.88
	CapZIC-HILIC-MS	A= 20332 C - 675	1 - 10	1.0	7.3	0.43
	CE-UV	A= 4.62 C + 0.40	1 - 20	1.0	5.3	1.3
	CE-MS	A= 2945 C - 9472	5 - 20	5.0	7.5	4.1

[a] The LOD was estimated by analyzing standard mixtures until 0.2 µM. The indicated value is the lowest concentration presenting a signal-to-noise ratio (S/N) close to and higher than 3, n=3).

Table 3. Separation performance for iso-16 and miR-21 standards at 10 μM and serum samples spiked at 2 μM by capZIC-HILIC-UV, capZIC-HILIC-MS, CE-UV and CE-MS. (n=3).

Technique	R_s (%RSD) [a]	N (%RSD) ($/10^3$) [b]	
		iso-16	miR-21
CapZIC-HILIC-UV (standards)	1.4 (4)	12 (2)	5.0 (4)
CapZIC-HILIC-MS (standards)	0.44 (14)	2.9 (17)	1.4 (14)
CapZIC-HILIC-MS (serum)	0.30 (27)	1.0 (20)	0.40 (24)
CE-UV (standards)	1.4 (4)	815(5)	680 (1)
CE-MS (standards)	0	1.2 (5)	1.6 (5)
CE-MS (serum)	0	1.2 (8)	1.0 (10)

[a] Separation resolution
[b] Number of theoretical plates

Supporting Information

Comparison of capillary electrophoresis and zwitterionic-hydrophilic interaction capillary liquid chromatography with ultraviolet and mass spectrometry detection for the analysis of microRNA biomarkers

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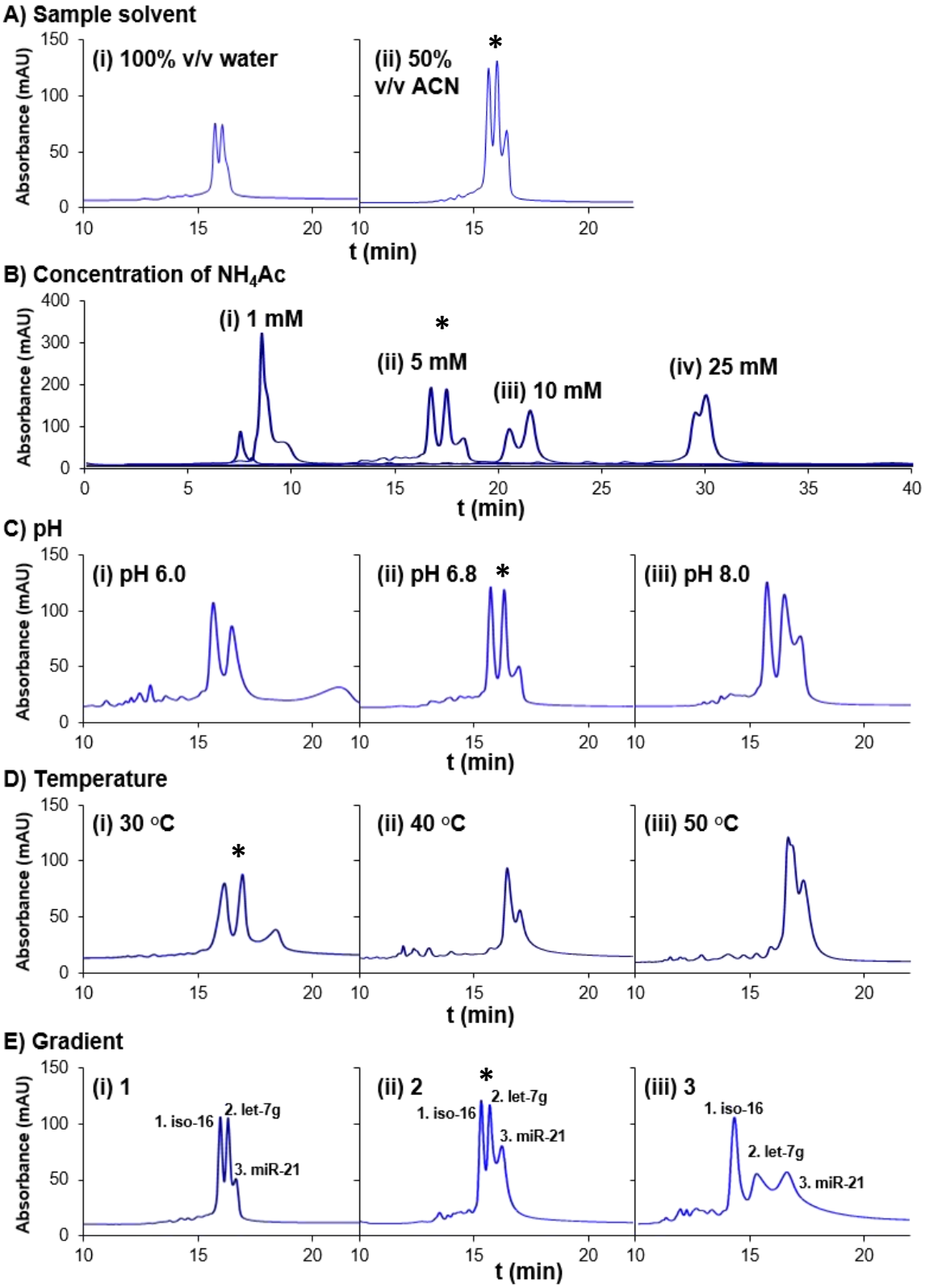

Figure S-1. Optimization of the separation of a 20 µM miRNA standard mixture of iso-16, let-7g, and miR-21 by capZIC-HILIC-UV. Selected conditions are indicated with an asterisk.

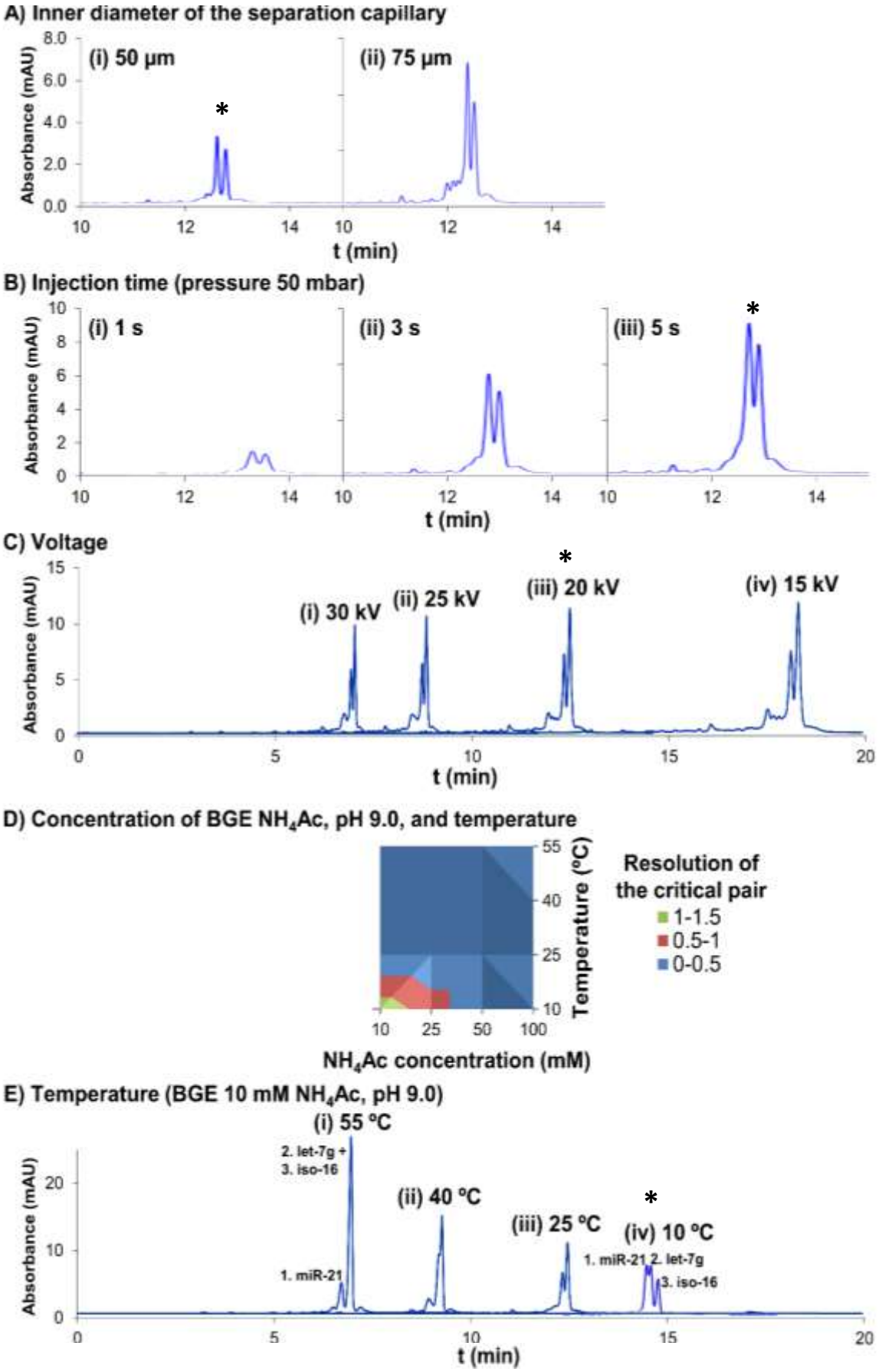

Figure S-2. Optimization of the separation of a 5 µM standard mixture of iso-16, let-7g, and miR-21 by CE-UV. Selected conditions are indicated with an asterisk.