SUPPORTING INFORMATION

Facile analysis of metabolites by capillary electrophoresis coupled to matrix-assisted laser desorption/ionization mass spectrometry using target plates with polysilazane nanocoating and grooves

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Experimental details

Materials

Benzylamine was purchased from TCI (Eschborn, Germany); acetyl-coenzyme A trilithium salt (Acetyl-CoA), adenosine 5'-diphosphate monosodium salt dihydrate (ADP), 9-aminoacridine (9-AA), β-nicotinamide adenine dinucleotide trihydrate (NAD), β-nicotinamide adenine dinucleotide disodium salt hydrate reduced form (NADH) and ±-verapamil hydrochloride from Acros Organics (Geel, Belgium); acetone, acetonitrile, adenosine 5'-triphosphate magnesium salt (ATP), ammonium bicarbonate, angiotensin I, angiotensin II acetate, α-cyano-4-hydroxy-cinnamic acid (CHCA), guanosine 5'-diphosphate sodium salt (GDP), H-Leu-Trp-Met-Arg-OH, sodium dihydrogen phosphate, trifluoroacetic acid (TFA) and Trizma base from Sigma-Aldrich (Buchs, Switzerland); sodium hydoxide from Siegfried Handel (Zofingen, Switzerland); ammonium acetate from Merck (Dietikon, Switzerland); acetic acid glacial from Carlo Erba Reagents (Rodano, Italy); ethanol from Scharlau Chemie (Sentmenat, Spain); methanol from Fisher Scientific (Loughborough, UK).

Stock solutions 10.0 mg mL^{-1} of NAD, NADH, Acetyl-CoA, ADP, ATP, GDP and verapamil salts, and 9.81 mg mL^{-1} benzylamine base were prepared in 18 MΩ cm nanopure water while angiotensin I, angiotensin II and H-Leu-Trp-Met-Arg-OH in a 1:1 (v/v) mixture of methanol and water. The model analyte mixture used for CE/MS method development was prepared by mixing 50 µL of stock solutions of the above-mentioned compounds, except for verapamil and benzylamine solutions (20 µL), as well as 50 µL of acetone (used as an EOF marker during method development), 250 µL of 20 mM ammonium acetate and 210 µL of nanopure water. Therefore, the molar concentrations were as follows: 1.83 mM benzylamine, 0.407 mM verapamil, 0.697 mM NAD, 0.705 mM NADH, 0.604 mM Acetyl-CoA, 1.03 mM

ADP, 0.941 mM ATP, 1.03 mM GDP, 0.386 mM angiotensin I, 0.478 mM angiotensin II and 0.827 mM H-Leu-Trp-Met-Arg-OH.

For the final testing of the developed method, a sample rich in biological matrix was prepared. It contained 2 mg mL^{-1} broth of Type I yeast (Sigma-Aldrich) and all the analytes as in the model analyte mixture mentioned above. To increase the amount of potential interferences in the sample and inactivate the enzymes that could potentially decompose the added standard compounds, the yeast broth was initially sonicated for 30 min and heated up to 95°C for 10 min. The molar concentrations of the spiked standards were as follows: 1.99 mM benzylamine, 0.444 mM verapamil, 0.759 mM NAD, 0.768 mM NADH, 0.658 mM Acetyl-CoA, 1.12 mM ADP, 1.03 mM ATP, 1.12 mM GDP, 0.420 mM angiotensin I, 0.521 mM angiotensin II and 0.901 mM H-Leu-Trp-Met-Arg-OH.

The 9-AA solution was normally prepared by dissolving 6.8 mg of 9-AA in 1 mL of an acetonitrile/acetone 2:1 (v/v) mixture. The CHCA solution was prepared by dissolving 10 mg of CHCA in 1 mL of an acetonitrile/water/TFA 50:50:0.1 (v/v/v) mixture.

Polyimide-coated fused silica capillary (50 μm i.d., 363 μm. o.d.) manufactured by Polymicro was purchased from BGB Analytik (Boeckten, Switzerland).

CE/deposition apparatus

In this study, a recycled XY-plotter (ZSK 2; Rohde & Schwarz, Ittigen, Switzerland) was used for deposition of effluent onto MALDI plates. The analog X-input of the plotter was connected to a function generator (DS335; Stanford Research Systems, Renens, Switzerland) (**Figure 2-S**). The X-input range of the plotter, the frequency and the amplitude of the triangle function were preset, so as to achieve the desired linear deposition speed (normally, 6.6 mm min^{-1}).

The pen holder of the plotter was modified in order to enable stable attachment of the free capillary ends as well as manual adjustment of the gap between the capillary tip and the writing plane. A miniature CMOS camera (size: 15 × 22 mm, lens: f=3.6mm, 250K (NTSC)) and a high intensity LED diode were mounted on a custom-designed metal holder attached in the pen position of the plotter. This enabled real time monitoring necessary for the optimization of the deposition process. A sheet of aluminum foil was attached to the surface of the writing plane of the plotter with adhesive tape. The aluminum foil was grounded, therefore the stainless steel MALDI plate laying on the top of it acted as a counter electrode during the electrophoretic separation (**Figure 2-S**).

The capillary used for separation needs to be long enough to enable free movement of the writing mechanism of the plotter. Normally, the length of 50 cm was sufficient to fulfill this condition. However, for the present study a capillary of 75 cm was chosen. The sensor head of the ActiPix D100 UV imaging detector (ActiPix D100; Paraytec, York, UK) was mounted ~15 cm above the writing plane and positioned 50 cm from the capillary inlet; giving a 50 cm effective capillary length and a 25 cm section from the detection window to the outlet end. The UV imaging detector was used for the monitoring of separation in parallel capillaries at a wavelength of 200±5 nm.

The inlet section of the capillary was mounted in a protective box fabricated of polycarbonate. The box housed a vial holder incorporating an HPLC vial cap (9 mm, with rubber/PTFE septum; 29044-U; Supelco, Bellefonte, PA, USA) and provided a connection for a high voltage power supply and the tubing to deliver overpressure. The platinum wire (0.5 mm), PEEK tubing (connected to the pressure system) and the capillaries were passed through the rubber/PTFE septum of the vial. This system allowed for fast and easy hydrodynamic

injection and change of vials (12 × 32 mm; 29000-U; Supelco) containing buffers and samples.

The high voltage power supply (ARM-30; Bertan, Hauppauge, NY, USA) was fitted with a home-built controller and housed in a separate casing. The system enabled application of potentials in the range from -30 to +30 kV and provided continuous monitoring of electric current. A lab-made pressure system was used to control the supply of overpressure during the injection, separation and capillary conditioning. An overpressure of 1 bar (≈ 100 kPa) was used for flushing the capillaries with 1 M NaOH, water and background electrolyte prior to separation. A 'low' overpressure of nitrogen, typically 40 mbar (≈ 4 kPa), was used for the injection and separation/deposition steps. A sphygmomanometer gauge allowed for precise control of this overpressure.

The temperature was kept constant by means of a thermostatting system. The capillary electrophoresis and deposition setup are housed in a thermally-insulated polycarbonate box and thermostatted with a Peltier device. Thermal insulation was improved with styrofoam sheets. An air-air thermoelectric cooler assembly (AA-040-12-22; Minco, Wil, Switzerland) was fitted with Peltier controller (TC2812; CoolTronic, Rossrüti, Switzerland) and a temperature sensor (S458PDZ12; Minco), and mounted in the deposition chamber. Additional fans were mounted to provide adequate air circulation in the main compartment as well as in the adjacent box housing the capillary inlet and the electrolyte vial (**Figure 2-S**). This system is thought to provide adequate temperature control on the whole capillary length, including the UV detection window, the inlet buffer reservoir as well as the effluent getting into physical contact with the MALDI plate.

Development of 3-D MALDI plates

While the preliminary experiments involved use of commercial MALDI plates, further development of the off-line CE-MALDI coupling imposed the need for customizing the plates. Therefore, the stainless steel MALDI plates used in further experiments were fabricated in-house. Microgrooves were machined along such plates in order to facilitate reception of the effluent from capillary. The grooves had a depth of 100 μm and a width of 200, 300, 400 or 500 μm. The cross-section of the grooves was either rectangular or triangular. The channel length was at least 10 cm, which allowed to deposit all the analytes coming out from the capillary within the preset time. The CE-MALDI plates, as described here, were fabricated either in the actual format of a standard plate fitting a commercial MALDI-MS instrument or as removable inserts, screwed onto a modified plate in the case of the Bruker Daltonics instrument or attached to the plate holder with double sided copper tape in the case of the Applied Biosystems instrument.

To enhance robustness of both steps, dispensing of MALDI matrix solutions and continuous deposition of CE effluent, a functional coating was applied to the surface of a blank plate *prior* to machining the grooves. Several commercial coatings, including PFA, PTFE and PEEK/fluoropolymer-mix, were screened for suitability. Eventually, a polysilazane nanocoating with a thickness of 5-10 μm (EpoTec 611/2320; Eposint, Pfyn, Switzerland; coating CAG 37 marketed by Clariant Produkte, Frankfurt am Main, Germany) was selected for the purpose of this study.

Before deposition of CE effluent, 100 μL of the 9-AA or 30 μL of the CHCA solutions (in 10 μL-aliquotes) were distributed along grooves using micropipette.

Mass spectrometry

A MALDI-TOF-MS instrument (Axima; Shimadzu/Kratos Analytical, Reinach, Switzerland) equipped with a 337 nm nitrogen laser and controlled with Launchpad/Kompact software (ver. 2.4.1; Kratos Analytical) was used for the initial method development. Typically, 2 shots were accumulated per each subspectrum and 181 subspectra were recorded during the readout of each bin corresponding to 4.5 mm distance along the deposition lane. Considering that the diameter of the laser spot is ~100 μm, and the gap between each pair of shots 25 μm, each point of the scanning path is illuminated at least 8 times with the laser.

At a later stage, the method was tested on two other MALDI-TOF instruments: an Ultraflex II (Bruker Daltonics, Billerica, MA, USA) and a MALDI-TOF/TOF-MS 4800 (Applied Biosystems, Foster City, CA, USA). In all cases, the rastering conditions were tailored using the available options of the data acquisition programs used. The MS readout of the deposition lane can potentially be fully automated to allow for a considerable increase of number of bins.

Data treatment

Mass spectra were acquired using the standard acquisition software packages supplied by the instrument manufacturers: Shimadzu/Kratos Analytical, Bruker Daltonics and Applied Biosystems. UV absorption electropherograms were registered using the ActiPix D100 software (2008 ver. 1.0.1120; Paraytec). For UV data treatment, an exponential filter with a time constant of 0.5 s was applied to all electropherograms. A cartridge file was initially created to accommodate signal registration with two parallel capillaries with the i.d. and o.d. used. Temperature control and image capture of the deposition were facilitated by TCCOM

(2007 build 1.6.4.101) and Ulead Video Studio (2006; Ulead Systems, Mountain View, CA, USA) software, respectively.

Two-column data files were handled using either OriginPro (2007 ver. v8.0725; OriginLab, Northampton, MA, USA) or Excel (2007 ver. 12.0.6331.5000; Microsoft, Redmond, WA, USA) software packages. For quantification purposes, peaks were fitted with a Haarhoff-Van der Linde (HVL) function and integrated using PeakFit software (2003 ver. 4.12; SeaSolve Software, Framingham, MA, USA). The raw MS spectra were normally exported to ASCII files and further analyzed using MATLAB software (2008 ver. 7.6.0.324 (R2008a); MathWorks, Natick, MA, USA). First, all the ASCII files corresponding to a single CE run were loaded and displayed as a bidimensional shaded surface plot using the MATLAB commands *surf* and *view*. The color map was defined by a logarithmic function with all the parameters selected in a way to obtain optimum contrast between the analyte peaks and the background. Another function was created to perform linear alignment of the time base in the 2-D CE/MS electropherogram (bins) against the UV electropherogram (seconds). Linear scaling was also applied to UV electropherograms obtained during separation in parallel capillaries; in this case, migration times of two well-defined bands at the beginning and at the end of the separation were used to calculate the scaling factors.

Additional information

For analysis of small molecules, a separation step before MS can be used (i) to obtain auxiliary information useful during identification, (ii) to separate species generating similar peaks (*e.g.* isobaric ions), (iii) to decrease ion suppression (sample matrix effects), (iv) to obtain information on the native state of the analyte (*e.g.* complexes), (v) to match gas-phase

ion adducts with the native state of analytes, or (vi) to reduce the number of false positives due to persistent ion peaks.

In the present study, we propose use of 3-D-patterned MALDI target plates with a polysilazane nanocoating and linear indents in the surface, introduced (i) to facilitate precoating of the deposition lane with MALDI matrix, (ii) to enable parallel continuous transfer of the CE effluent onto the plate during separation in a way that prevents dispersion of the analytes, and (iii) to facilitate multiple readout of the deposited traces using commercial MALDI instruments. In addition, this approach has several other advantages, for example: (i) Conditioning of the CE capillary is straightforward – the deposition tip is simply moved to a position outside the MALDI plate and the capillary can be flushed with sodium hydroxide or other solutions as required. (ii) The whole system can easily be thermostatted to increase ruggedness of the separation and the interface. (iii) Plates with separated samples can be stored frozen for a later analysis. This is relevant for high throughput operation of MALDI-MS instruments as well as for archiving. (iv) This approach also enables recycling of vintage electronic equipment (XY-plotters and low frequency function generators) to construct and operate easy-to-use analytical instrumentation.

The method presented here delivers inexpensive automation for researchers in biochemistry and as such should be reliable. As indicated, it has some tolerance to misalignment of the capillary end section and the groove axis. Unlike in other studies on continuous deposition, the capillary end was held perpendicular to the deposition plane, which eliminated the need to optimize and control the deposition angle.

Beneficial properties of polysilazane nanocoating are: (i) low wettability (for aqueous and organic solvent solutions), (ii) adhesion to stainless steel surface, (iii) chemical inertness, (iv) no significant ionization in the MALDI process, (v) durability, (vi) no evident porosity.

The presented plate design, incorporating the polysilazane nanocoating and grooves, together with the straightforward deposition scheme can potentially be used with other methods, for example, in-capillary chromatographic separation before and/or non-MS spectroscopic readout after the deposition.

Additional figures

Figure 1-S. MALDI-MS spectra of standard solutions of NAD and NADH. The deprotonated molecule could not be detected for NAD while isobaric fragments (*m/z*: 426 and 540) are detected in both cases.

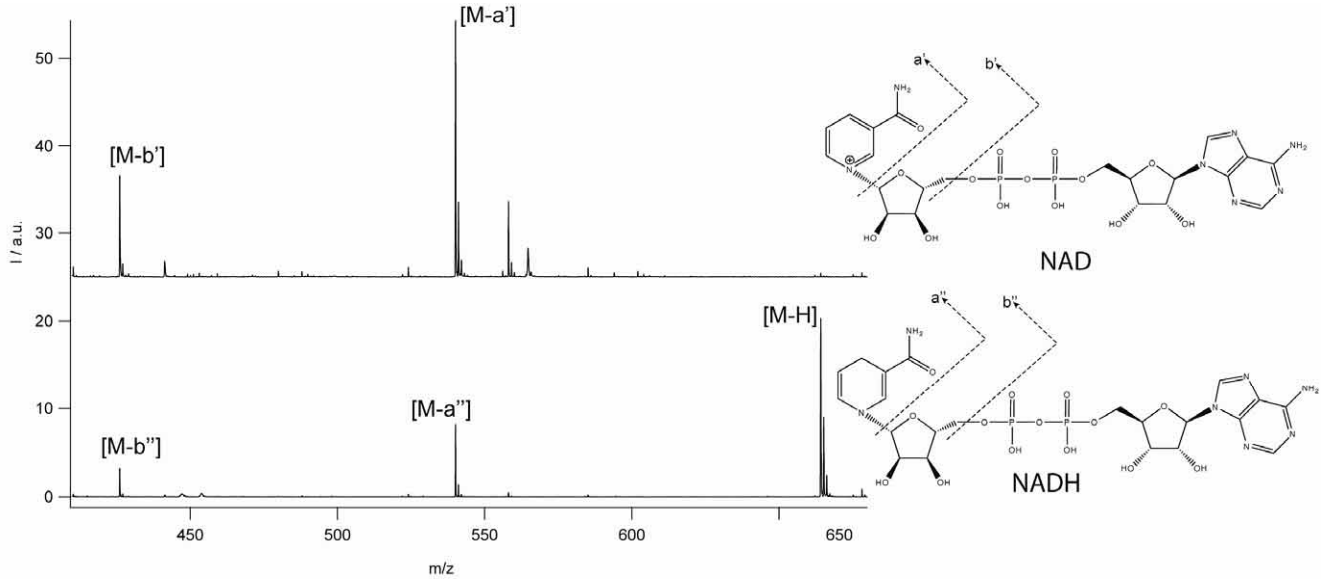


Figure 2-S. Photograph of the CE and deposition apparatus.

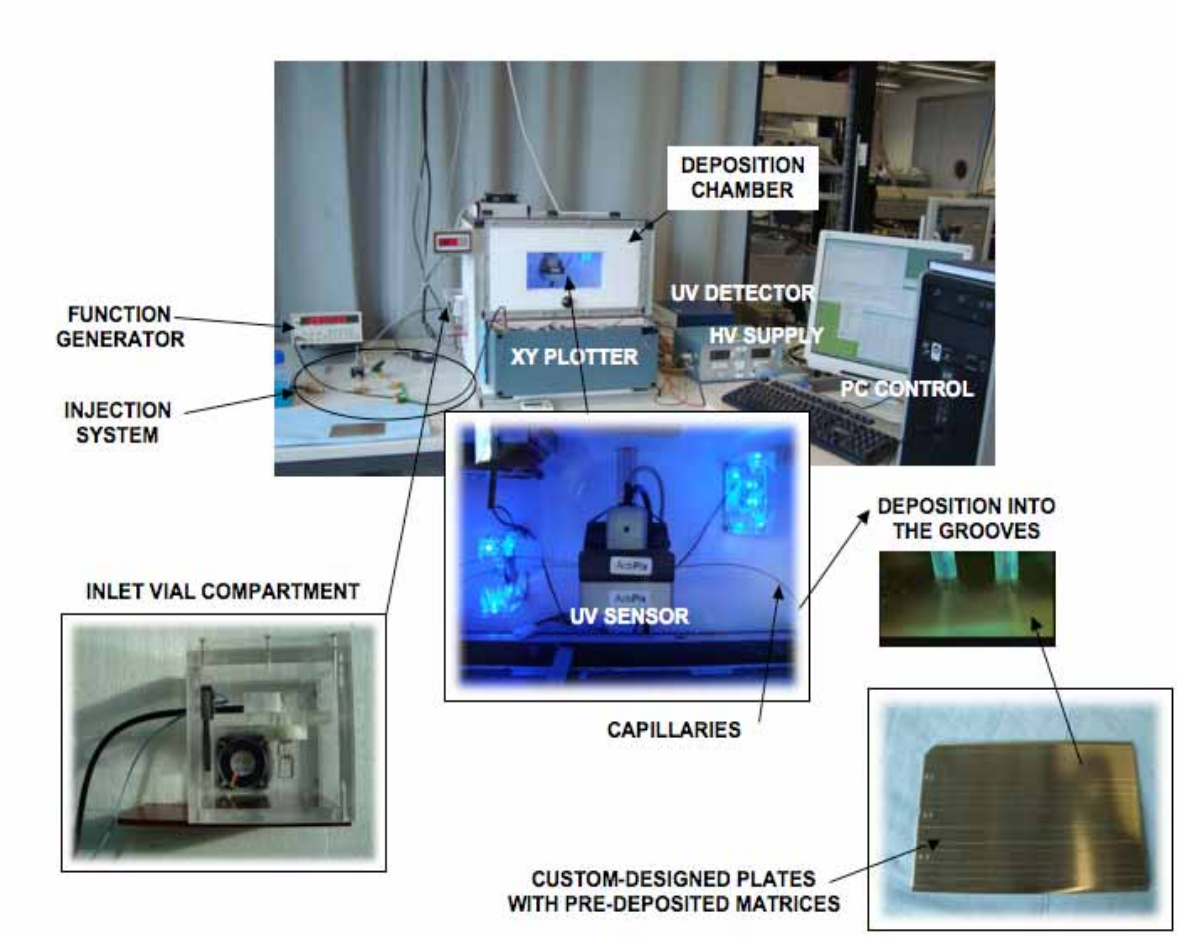


Figure 3-S. Separation of standard compounds by CE with UV absorption detection. The test sample was prepared by mixing the stock solutions: 25 µL benzylamine, 25 µL verapamil, 50 µL NAD, 50 µL NADH, 50 µL Acetyl-CoA as well as 250 µL 20 mM ammonium acetate, 50 µL acetone (EOF marker) and 500 µL nanopure water.

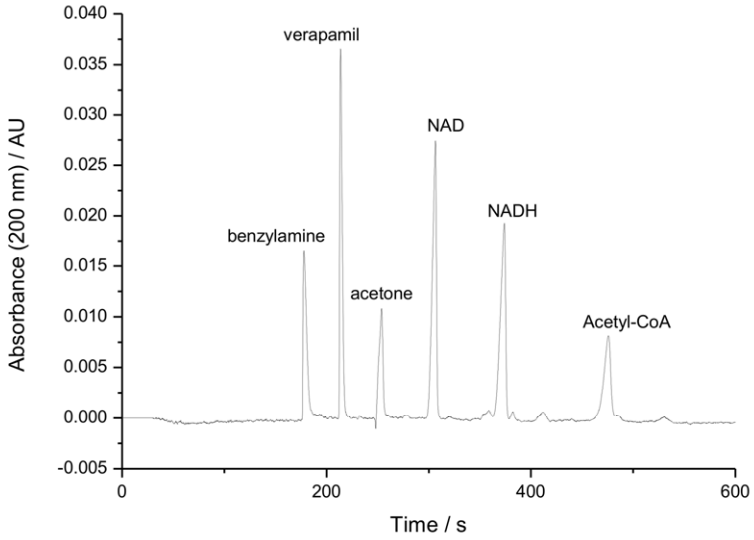
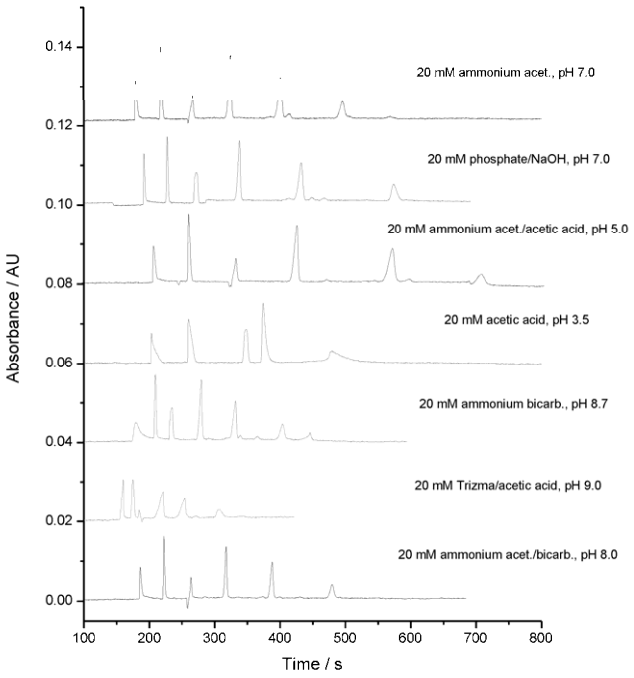
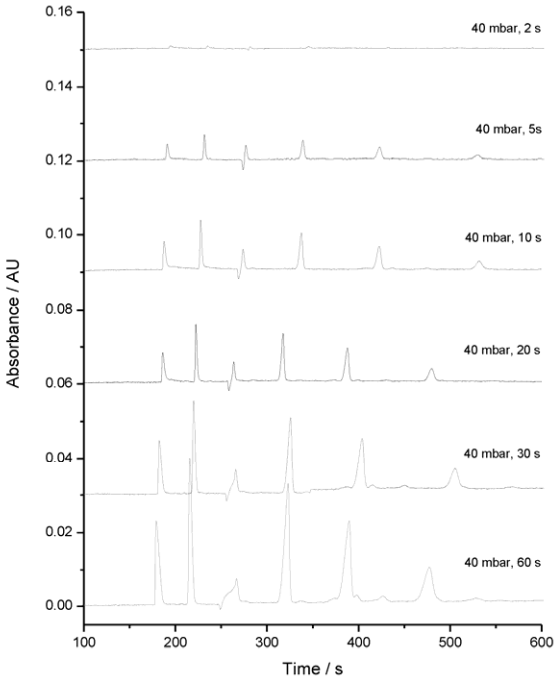


Figure 4-S. Optimization of the CE method (without deposition). Effect of: (A) type of background electrolyte, (B) time of applying overpressure during hydrodynamic injection, (C) separation voltage and temperature, and (D) additional pressure applied during separation. Basic CE conditions (unless indicated otherwise): polyimide-coated fused silica capillary (length 75 cm (50 cm from the inlet to the UV detector), i.d. 50 μm, o.d. 363 μm); hydrodynamic injection, 40 mbar 20 s; separation, +20 kV (pressure applied to the inlet vial during separation, +40 mbar); background electrolyte: 20 mM CH_3COONH_4 adjusted to pH 8.0 with 1 M NH_4HCO_3; UV detection at 200 nm; thermostat temperature: 25°C. Sample as in **Figure 3-S**, diluted 1:1 with nanopure water. Peak assignment as in **Figure 3-S**.

(A)

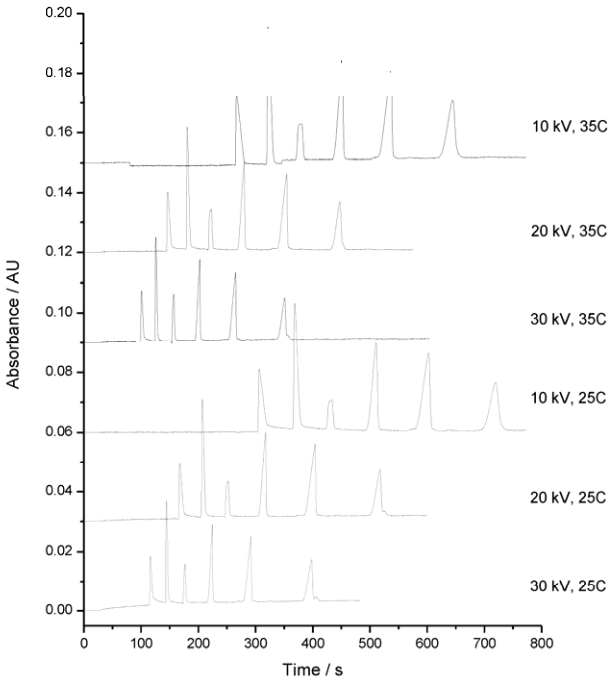


1 **(B)**


40 mbar, 2 s
40 mbar, 5s
40 mbar, 10 s
40 mbar, 20 s
40 mbar, 30 s
40 mbar, 60 s
Absorbance / AU
Time / s

2

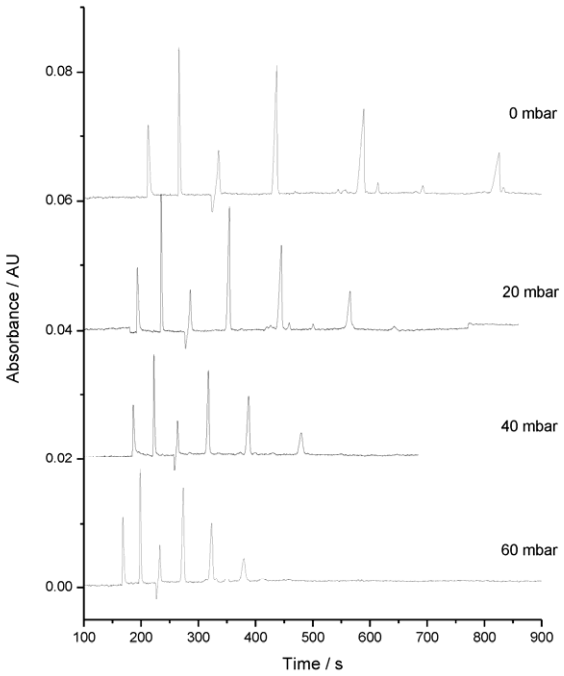
3 **(C)**


10 kV, 35C
20 kV, 35C
30 kV, 35C
10 kV, 25C
20 kV, 25C
30 kV, 25C
Absorbance / AU
Time / s

4

5

1 **(D)**


0.08
0.06
0.04
0.02
0.00
Absorbance / AU
0 mbar
20 mbar
40 mbar
60 mbar
100
200
300
400
500
600
700
800
900
Time / s

2

3