Video Article

Sheathless Capillary Electrophoresis–Mass Spectrometry for Metabolic Profiling of Biological Samples

Wei Zhang[1], M. Can Gulersonmez[1], Thomas Hankemeier[1], Rawi Ramautar[1]

[1]Division of Analytical Biosciences, Leiden Academic Center for Drug Research, Leiden University

Correspondence to: Rawi Ramautar at r.ramautar@lacdr.leidenuniv.nl

Abstract

In metabolomics, a wide range of analytical techniques is used for the global profiling of (endogenous) metabolites in complex samples. In this paper, a protocol is presented for the analysis of anionic and cationic metabolites in biological samples by capillary electrophoresis–mass spectrometry (CE-MS). CE is well-suited for the analysis of highly polar and charged metabolites as compounds are separated on the basis of their charge-to-size ratio. A recently developed sheathless interfacing design, *i.e.,* a porous tip interface, is used for coupling CE to electrospray ionization (ESI) MS. This interfacing approach allows the effective use of the intrinsically low-flow property of CE in combination with MS, resulting in nanomolar detection limits for a broad range of polar metabolite classes. The protocol presented here is based on employing a bare fused-silica capillary with a porous tip emitter at low-pH separation conditions for the analysis of a broad array of metabolite classes in biological samples. It is demonstrated that the same sheathless CE-MS method can be used for the profiling of cationic metabolites, including amino acids, nucleosides and small peptides, or anionic metabolites, including sugar phosphates, nucleotides and organic acids, by only switching the MS detection and separation voltage polarity. Highly information-rich metabolic profiles in various biological samples, such as urine, cerebrospinal fluid and extracts of the glioblastoma cell line, can be obtained by this protocol in less than 1 hr of CE-MS analysis.

Video Link

The video component of this article can be found at https://www.jove.com/video/54535/

Introduction

In contemporary metabolomics, high end analytical separation techniques are used to analyze a wide range of metabolite classes in order to obtain a representative read-out of the physiological status of an organism[1]. The ultimate objective of a metabolomics study is to obtain an answer to a given biological/clinical question. At present, the Human Metabolome Database is comprised of more than 40,000 metabolite entries representing both endogenous and exogenous compounds (the latter originating from nutrients, microbiota, drugs and other sources)[2]. Given the huge diversity in physico-chemical properties and concentration range of these metabolites, multiple analytical techniques with different separation mechanisms should be used in conjunction in order to profile as many metabolites as possible in a given biological sample. For example, Psychogios *et al.* used a combination of five analytical separation techniques for metabolic profiling of human serum resulting in the detection of more than 4,000 chemically diverse metabolites[3].

In this paper, attention will be paid to recently developed CE-MS strategies for metabolic profiling of biological samples[4,5]. In CE, more specifically capillary zone electrophoresis (CZE; normally referred as CE), compounds are separated on the basis of their charge-to-size ratio and, therefore, this analytical technique is highly suited for the analysis of polar and charged metabolites. The separation mechanism of CE is fundamentally different from chromatographic-based techniques, thereby providing a complementary view on the metabolic composition of biological samples[6-8]. Soga and co-workers were the first to show the utility of CE-MS for the global profiling of metabolites in biological samples[9,10]. Until now, the feasibility and usefulness of CE-MS for metabolomics has been widely demonstrated[11-15]. CE is generally coupled to MS via a sheath-liquid interfacing technique[16,17]; however, due to dilution of the capillary effluent by the sheath-liquid, the detection sensitivity is intrinsically compromised.

Recently, it was demonstrated that the use of a sheathless interface significantly improved the detection coverage of metabolites present in various biological samples as compared to CE-MS utilizing a classical sheath-liquid interface[5,18,19]. For example, circa 900 molecular features were detected in human urine by sheathless CE-MS whereas about 300 molecular features were observed with sheath-liquid CE-MS[5]. The sheathless interface used was based on a porous tip emitter, which was invented by Moini[20], allowing the effective use of the intrinsically low-flow property of CE in combination with nano-ESI-MS.

In order to stimulate the use of sheathless CE-MS in the field of metabolomics, a protocol is presented describing how this approach can be used for the analysis of highly polar metabolites in biological samples, as exemplified for the analysis of extracts from the glioblastoma cell line. It is shown that the sheathless CE-MS method for the profiling of cationic metabolites can also be used for the profiling of anionic metabolites using

exactly the same capillary and separation conditions, thereby reducing analysis time and providing one single analytical platform for the global profiling of charged metabolites. The protocol also describes a strategy for the effective alignment of the sheathless porous tip emitter with the MS instrument.

Protocol

NOTE: The protocol described here for the use of sheathless CE-MS for metabolic profiling studies is for laboratory use only. The procedures outlined below are based on recently published work[4,5]. Further experimental details can be found in these papers. Prior to using this protocol, consult all relevant material safety data sheets (MSDS). Please use all appropriate laboratory safety procedures, including safety glasses, lab coat and gloves, when conducting the experiments outlined in this protocol.

1. Preparation of Reagents Solutions and Samples

1. **Preparation of the background electrolyte (BGE)**
    1. Prepare a new BGE solution (10% (v/v) acetic acid, pH 2.2) every day.
    2. Add 9.0 ml of water into a 10 ml glass vial and add 1.0 ml of acetic acid to the water in a fume hood. Mix the solution thoroughly using a vortex.

2. **Preparation of metabolite standard mixture**
    1. Dissolve 50 μl of a 50 μM cation standard mixture containing 60 cationic metabolites into 50 μl of water and mix the solution thoroughly. Store at -80 °C when not in use.
    2. Dissolve 50 μl of a 50 μM anion standard mixture containing 30 anionic metabolites into 50 μl of water and mix the solution thoroughly. Store this solution, in aliquots to prevent freeze/thaw cycles of the same standard mixture, at -80 °C when not in use.
    NOTE: The metabolite standard mixtures are stable for 3 months when stored properly at -80 °C.

3. **Preparation of extracts from the glioblastoma cell line**
    1. Wash the adherent human U-87 MG glioblastoma cells three times with 1 ml ice-cold 0.9% sodium chloride solution[21].
    2. Add 2 ml ice-cold methanol/water solution (8/2, v/v) to the adherent cells and scrape using a rubber tipped cell scraper[21].
    3. Collect the methanol/water solution in a tube and ultrasonicate for 2 min.
    4. Add chloroform to the methanol/water fraction (final ratio 8/8/2, v/v/v) and centrifuge the sample for 10 min at 16,100 x g and 4 °C.
    5. Collect the methanol/water layer and evaporate this fraction using a vacuum concentrator. Reconstitute the dried material in 50 μl water for analysis by sheathless CE-MS. When not in use store the sample at -80 °C.

2. Setting up the Sheathless CE-MS System

1. **Installation of the bare fused-silica cartridge with the porous tip emitter**
    1. Place a new bare fused-silica cartridge with a porous tip emitter (30 μm inner diameter x 90 cm total length) in the CE instrument.
    2. Apply a forward rinse at 50 psi for 15 min with the software controlling the CE instrument using 100% methanol and visually check whether liquid is flowing out from the capillary outlet during this rinsing step. Also perform a rinse in the opposite direction at 50 psi for 5 min using the BGE solution to visually examine whether liquid is flowing out from the conductive capillary.
    3. Repeat step 2.1.2 at a pressure of 100 psi in case no liquid drop formation has been observed at the capillary outlet. Install a new bare fused-silica cartridge if no liquid drop formation was observed during this step.
    4. Rinse the separation capillary with water at 50 psi for 10 min, followed by 0.1 M NaOH at 50 psi for 10 min, then by water at 50 psi for 10 min and finally with BGE at 50 psi for 10 min.
    NOTE: Steps 2.1.1 until 2.1.4 are only required for the installation of a new capillary cartridge.

2. **Coupling of the capillary porous tip emitter to ESI-MS**
    NOTE: Prior to coupling the CE capillary to MS, ensure that the MS instrument has been calibrated and connected to the CE system. Set the ESI voltage to 0. Fit the MS instrument with a nanospray source. Gas 1, gas 2 and interface heater temperature were not applied as ESI at very low flow rates occurs by just applying the ion spray voltage set at 1500 V. Set the curtain at 5 psi.
    1. Remove the sprayer tip of the fused-silica cartridge from the water tube and install it in the nanospray source adapter for coupling to the MS instrument. Ensure that the height of the BGE vials in the CE instrument match the height of the sprayer tip.
    2. Check for flow of liquid through the conductive capillary by rinsing with BGE at 50 psi for 5 min. During this rinsing step a drop formation at the base of the ESI sprayer needle should be observed.
    3. Flush the separation capillary with BGE at 50 psi for 10 min in the forward direction. Drop formation should be observed at the tip of the porous tip emitter (sprayer tip) during this step.
    4. Position the porous tip emitter to the entrance of the MS inlet at a distance of circa 2 to 3 mm.
    5. Apply a voltage of 30 kV using a ramp time of 1 min and start acquiring MS data in the *m/z* range from 65 to 1,000 *m/z* for metabolic profiling studies using first an ESI voltage of 0.
    NOTE: The mass spectrum should be void of signal as there should be no electrospray.
    6. Set the ESI voltage to 1,000 V while carry on measuring data. Increase the ESI voltage with increments of 200 V until a constant background signal is observed for at least 15 min.
    7. Optimize the porous tip emitter position with respect to the center of the MS inlet by moving it in the x, y, or z-direction in order to see which position provides the maximal and most stable MS signal (total ion electropherogram).
    8. After optimizing the position of the porous tip emitter and determining the optimal ESI voltage, set the ESI voltage to 0 V and decrease the CE voltage from 30 kV to 1 kV using a ramp time of 5 min.

9. Create a MS method using the optimal ESI voltage and a CE method on the CE instrument for the analysis of metabolite standards and biological samples.

3. Analysis of Metabolite Standards and Biological Samples

1. **Performance evaluation of the sheathless CE-MS system**
    1. Transfer 20 µl of the anionic metabolite standard mixture into an empty 100 µl microvial (PCR vial) which fits into a CE vial and put this vial in the inlet sample tray.
       NOTE: The minimum volume required in the microvial for a reliable injection is 2 µl.
        1. Start the MS acquisition negative ion mode method created during step 2.2.9 and subsequently start the CE sequence using the software controlling the CE instrument.
        2. Rinse the separation capillary with BGE at 50 psi for 3 min followed by injection at 2.0 psi for 60 sec (20 nl corresponding to 3% of the capillary volume) and then by BGE injection at 1.0 psi for 10 sec.
           NOTE: Subsequently, MS data acquisition is triggered.
        3. Apply a voltage of -30 kV with a ramp time of 1.0 min with a pressure of 0.5 psi for 30 min at the inlet. After a 30 min electrophoretic separation, stop MS data acquisition and decrease the CE voltage to -1 kV using a ramp time of 5 min (a gradual decrease of the CE voltage after the electrophoretic separation improves the durability of the porous tip capillary emitter).
    2. Between sample injections, rinse the capillary with water, 0.1 M sodium hydroxide, water and BGE each at 30 psi for 3 min.
    3. Analyze the recorded data by determining the migration times and the signal intensity of the analyzed anionic metabolite mixture.
    4. Assess whether the anionic metabolite standards appear in the region between 10 and 28 min.
    5. Check whether three structurally related isomers, *i.e.*, D-Glucose-1-phosphate, D-Glucose-6-phosphate and D-Fructose-6-phosphate are partially separated, *i.e.*, the resolution between the first two peaks is circa 0.75 and of the last two peaks is circa 0.50 (see **Figure 1**).
       NOTE: A resolution of 1.5 indicates a baseline separation of two adjacent peaks.
   6. Repeat steps 3.1.1 and 3.1.2 for the cationic metabolite mixture. Ensure that MS detection is now in positive ion mode and CE voltage is +30 kV.
    7. Analyze the recorded data by determining the migration times and the signal intensity of the analyzed cationic metabolite mixture.
    8. Assess whether the cationic metabolite standards appear in the region between 8 and 22 min. Check whether isoleucine and leucine are migrating between 15 and 15.5 min and determine if the resolution is circa 0.5.

2. **Analysis of biological samples**
    1. Repeat steps 3.1.1 and 3.1.2 for anionic metabolic profiling of the extract of the glioblastoma cell line.
       NOTE: The metabolic content obtained after sample pretreatment corresponds to a cell density of circa 20 cells/nl, therefore, a 20 nl injection is the equivalent of 400 cells per analysis. .
    2. Create an extracted ion electropherogram for the metabolite lactic acid (*m/z* 89.0243) using 5 mDa mass accuracy and check whether the signal intensity is above 100,000 counts.
    3. Repeat steps 3.1.1 and 3.1.2 for cationic metabolic profiling of the extract of the glioblastoma cell line.
       NOTE: A highly information-rich total ion electropherogram should be observed for a 20 nl injection in this mode.
    4. After the analyses or when not in use, rinse the capillary with water at 50 psi for 15 min and store the inlet part of the capillary in a vial containing water and the porous section (outlet part) in a tube also containing water.

Representative Results

The proposed sheathless CE-MS method is capable of providing highly efficient, *i.e.,* plate numbers ranging from 60,000 to 400,000, profiles for anionic and cationic metabolites at nanomolar detection limits using 10% acetic acid (pH 2.2) as BGE. The separation performance of the method for the analysis of highly polar anionic metabolites is demonstrated for three structurally related sugar phosphate isomers (**Figure 1**). Though a baseline separation was not obtained for these three analytes, a partial separation is sufficient to allow their selective detection by MS as these analytes have the same exact mass. The potential of the sheathless CE-MS method for metabolic profiling of limited number of cells, *i.e.*, a 20 nl injection corresponds to 400 cells (cell density is circa 20 cells/nl), is demonstrated for the analysis of cationic metabolites in an extract of the glioblastoma cell line (**Figure 2**), in which more than 300 molecular features were detected above a S/N-ratio ≥ 5.
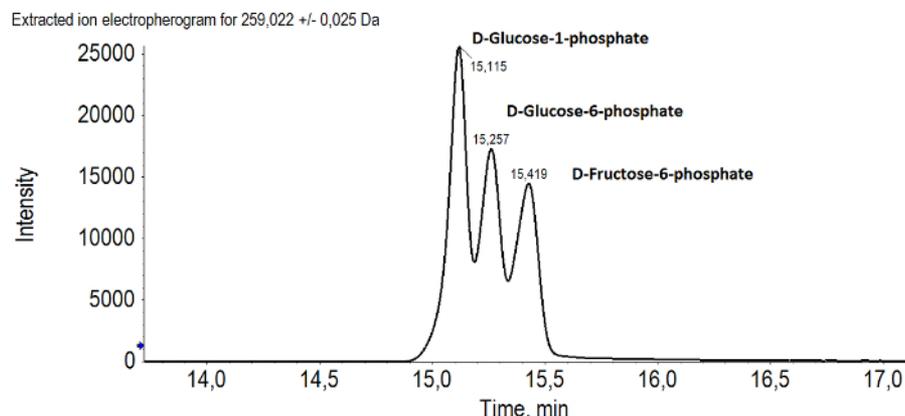
Figure 1. Analysis of sugar phosphate isomers by sheathless CE-MS. Extracted ion electropherogram for three sugar phosphate isomers (25 µM) obtained with sheathless CE-MS in negative ion mode. Experimental conditions: BGE, 10% acetic acid (pH 2.2); separation voltage, -30 kV (+0.5 psi applied at the inlet of the CE capillary); sample injection, 2.0 psi for 60 sec. Reproduced with permission[4]. Please click here to view a larger version of this figure.
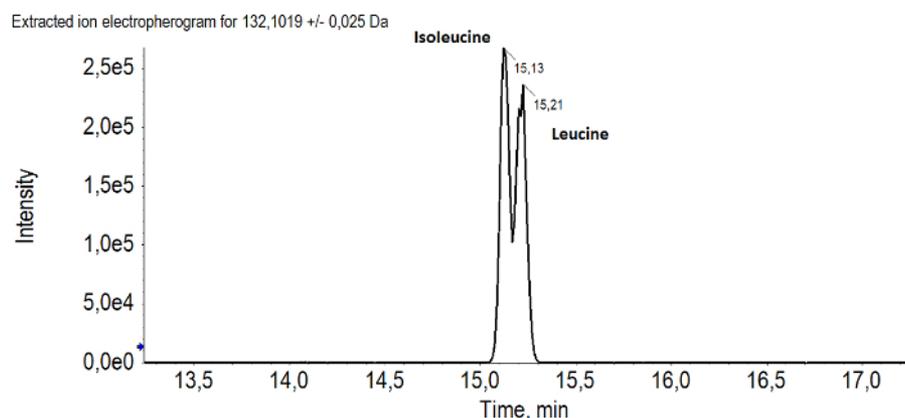
Figure 2. Analysis of isoleucine and leucine by sheathless CE-MS. Extracted ion electropherogram of two amino acid isomers (25 µM) obtained with sheathless CE-MS in positive ion mode. Experimental conditions: BGE, 10% acetic acid (pH 2.2); separation voltage, +30 kV; sample injection, 2.0 psi for 60 sec. Please click here to view a larger version of this figure.
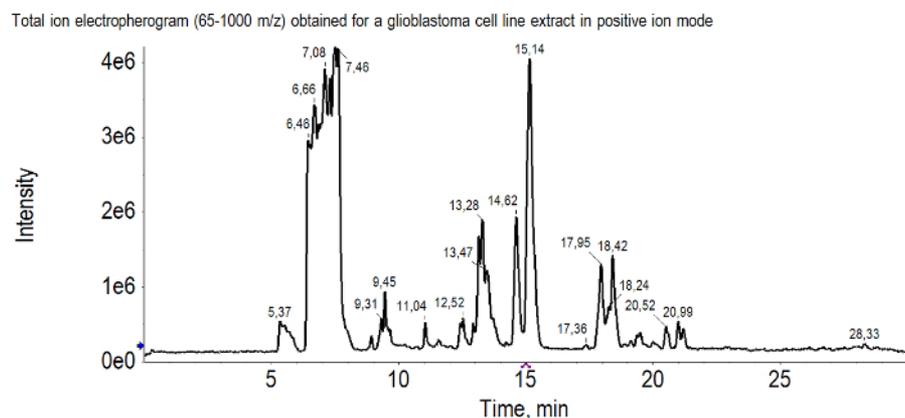
Figure 3. Potential of sheathless CE-MS for profiling cationic metabolites in a cell line extract. Metabolic profile (total ion electropherogram) observed in an extract of a glioblastoma cell line with sheathless CE-MS in positive ion mode. Experimental conditions: BGE, 10% acetic acid (pH 2.2); separation voltage, +30 kV; sample injection, 2.0 psi for 60 sec. Reproduced with permission[4]. Please click here to view a larger version of this figure.

Discussion

A sheathless CE-MS method employing a porous tip emitter has been presented for the analysis of highly polar and charged metabolites. A unique feature of this approach is that anionic or cationic metabolites can be profiled by only switching the MS detection and CE voltage polarity. A wide range of highly polar and charged metabolites in biological samples can be analyzed with a high separation efficiency, which is crucial for structurally similar metabolites, and with limits of detection in the (low) nanomolar range. The presented protocol focused on the use

of sheathless CE-MS for metabolic profiling of cell extracts in order to exemplify the utility of the method for metabolic profiling of a biological sample. The approach described here can also be used for metabolic profiling of other types of biological samples, such as human urine[5], given that a proper sample pretreatment procedure is used.

The sheathless CE-MS method is based on a porous tip emitter which allows the usage of the intrinsically low-flow property of CE. In this context, a stable ESI signal is a pre-requisite for reproducible metabolic profiling studies. Thus, it is important that the sprayer tip is properly positioned in front of the MS inlet. In this set-up, the ESI process is mainly dependent on the nature of the BGE and therefore, BGE optimization is critical. The sheathless configuration is less versatile in comparison to sheath-liquid CE-MS systems where all kinds of sheath-liquid compositions can be added to improve the ionization efficiency. The sheathless ESI sprayer needle needs to be completely filled with conductive liquid (i.e., BGE solution). An unstable ESI signal may result from a partial or fully plugged capillary. Rinsing at high pressures with BGE may solve this issue. Otherwise the separation capillary needs to be replaced. Prior to assessment of the analytical performance, a stable ESI background signal should be generated first which is consistent from one day to another.

The analytical performance of the sheathless CE-MS method for metabolic profiling studies needs to be checked daily using metabolite standard mixtures. Under the same experimental conditions, consistent migration times, i.e., variation below 3% for within-day (n=10) and between-day (n=5) using a 20 nl injection of a metabolite standard mixture (12.5 μM), peak heights/areas (variation below 15%) and plate numbers (ranging between 60,000 and 400,000) should be obtained. Limits of detection should be in the nanomolar range for most metabolite standards. Only when these criteria are met is the method ready for metabolic profiling of biological samples. If not, the MS instrument needs to be tuned and re-calibrated or the porous tip capillary emitter needs to be changed.

An effective rinsing step between CE-MS analyses is of high importance, not only to prevent potential carryover but also to maintain the separation performance. Potential carryover may be caused by BGE vials contaminated with the sample and therefore solved by replacement with new BGE vials. When the sheathless CE-MS method is not in use, it is important to disconnect the separation capillary and to store the inlet side of the capillary in water and the outside submerged with the protective sleeve in a tube containing water to prolong capillary lifetime.

In summary, the proposed sheathless CE-MS method shows a strong potential for metabolic profiling of biological samples when used according to the procedures reported in this protocol. At this stage, inter-laboratory comparison data are definitely needed for sheathless CE-MS in order to assess the (long-term) reproducibility and robustness of this approach for metabolomics. This protocol may stimulate such a study. Various analytical challenges still need to be considered. For optimal performance, the CE current should be kept preferably below 5 μA and at this stage the capillary porous tip emitters are only provided at a length of 91 cm which may hamper the development of high-throughput assays. Moreover, a low pH separation buffer was used for anionic metabolic profiling which may not be the most optimal for achieving a baseline separation of structurally related sugar phosphates. Also important is that only anionic metabolites can be analyzed which are (partially) negatively charged under the used separation conditions. The next step is to assess the utility of the sheathless CE-MS method for clinical metabolic profiling studies as currently, a single porous tip capillary emitter can only be used for the analysis of up to 100 biological samples.

Overall, further development in the sheathless CE-MS approach will open a new direction in the field of metabolomics, i.e., towards a deeper understanding of biological functions in sample-restricted cases.

Disclosures

We have nothing to disclose.

Acknowledgements

References

1. Ramautar, R., Berger, R., van der Greef, J., & Hankemeier, T. Human metabolomics: strategies to understand biology. *Curr Opin Chem Biol*. **17**, 841-846 (2013).
2. Wishart, D. S. *et al.* HMDB 3.0--The Human Metabolome Database in 2013. *Nucleic Acids Res* **41**, D801-807 (2013).
3. Psychogios, N. *et al.* The human serum metabolome. *PLoS One* **6**, e16957 (2011).
4. Gulersonmez, M. C., Lock, S., Hankemeier, T., & Ramautar, R. Sheathless capillary electrophoresis-mass spectrometry for anionic metabolic profiling. *Electrophoresis*. **37**, 1007-1014 (2016).
5. Ramautar, R., Busnel, J. M., Deelder, A. M., & Mayboroda, O. A. Enhancing the coverage of the urinary metabolome by sheathless capillary electrophoresis-mass spectrometry. *Anal Chem*. **84**, 885-892 (2012).
6. Ramautar, R. *et al.* Metabolic profiling of human urine by CE-MS using a positively charged capillary coating and comparison with UPLC-MS. *Mol Biosyst* **7**, 194-199 (2011).
7. Naz, S., Garcia, A., & Barbas, C. Multiplatform analytical methodology for metabolic fingerprinting of lung tissue. *Anal Chem*. **85**, 10941-10948 (2013).
8. Ibanez, C. *et al.* CE/LC-MS multiplatform for broad metabolomic analysis of dietary polyphenols effect on colon cancer cells proliferation. *Electrophoresis* **33**, 2328-2336 (2012).
9. Soga, T. *et al.* Simultaneous determination of anionic intermediates for Bacillus subtilis metabolic pathways by capillary electrophoresis electrospray ionization mass spectrometry. *Anal Chem* **74**, 2233-2239 (2002).
10. Soga, T. *et al.* Quantitative metabolome analysis using capillary electrophoresis mass spectrometry. *J Proteome Res* **2**, 488-494 (2003).
11. Britz-McKibbin, P. Capillary electrophoresis-electrospray ionization-mass spectrometry (CE-ESI-MS)-based metabolomics. *Methods Mol Biol*. **708**, 229-246 (2011).

12. Ibanez, C. *et al.* A new metabolomic workflow for early detection of Alzheimer's disease. *J Chromatogr A* **1302**, 65-71 (2013).
13. Kuehnbaum, N. L., & Britz-McKibbin, P. New advances in separation science for metabolomics: resolving chemical diversity in a post-genomic era. *Chem Rev.* **113**, 2437-2468 (2013).
14. Nemes, P., Rubakhin, S. S., Aerts, J. T., & Sweedler, J. V. Qualitative and quantitative metabolomic investigation of single neurons by capillary electrophoresis electrospray ionization mass spectrometry. *Nat Protoc.* **8**, 783-799 (2013).
15. Hirayama, A., Wakayama, M., & Soga, T. Metabolome analysis based on capillary electrophoresis-mass spectrometry. *Trac-Trend Anal Chem.* **61**, 215-222 (2014).
16. Maxwell, E. J., & Chen, D. D. Twenty years of interface development for capillary electrophoresis-electrospray ionization-mass spectrometry. *Anal Chim Acta.* **627**, 25-33 (2008).
17. Bonvin, G., Schappler, J., & Rudaz, S. Capillary electrophoresis-electrospray ionization-mass spectrometry interfaces: fundamental concepts and technical developments. *J Chromatogr A.* **1267**, 17-31 (2012).
18. Hirayama, A., Tomita, M., & Soga, T. Sheathless capillary electrophoresis-mass spectrometry with a high-sensitivity porous sprayer for cationic metabolome analysis. *Analyst.* **137**, 5026-5033 (2012).
19. Bonvin, G., Schappler, J., & Rudaz, S. Non-aqueous capillary electrophoresis for the analysis of acidic compounds using negative electrospray ionization mass spectrometry. *J Chromatogr A.* **1323**, 163-173 (2014).
20. Moini, M. Simplifying CE-MS operation. 2. Interfacing low-flow separation techniques to mass spectrometry using a porous tip. *Anal Chem.* **79**, 4241-4246 (2007).
21. Dettmer, K. *et al.* Metabolite extraction from adherently growing mammalian cells for metabolomics studies: optimization of harvesting and extraction protocols. *Anal Bioanal Chem* **399**, 1127-1139 (2011).