Capillary Electrophoresis-Mass Spectrometry for Metabolomics: Possibilities and Perspectives

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Abstract

Capillary electrophoresis-mass spectrometry (CE-MS) is a very useful analytical technique for the selective and highly efficient profiling of polar and charged metabolites in a wide range of biological samples. Compared to other analytical techniques, the use of CE-MS in metabolomics is relatively low as the approach is still regarded as technically challenging and not reproducible. In this chapter, the possibilities of CE-MS for metabolomics are highlighted with special emphasis on the use of recently developed interfacing designs. The utility of CE-MS for targeted and untargeted metabolomics studies is demonstrated by discussing representative and recent examples in the biomedical and clinical fields. The potential of CE-MS for large-scale and quantitative metabolomics studies is also addressed. Finally, some general conclusions and perspectives are given on this strong analytical separation technique for probing the polar metabolome.

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Keywords

Capillary electrophoresis · Mass spectrometry · Metabolomics · Interfacing designs · Applications

Abbreviations

APFO	Ammonium perfluorooctanoate
BGE	Background electrolyte
CEC	Capillary electrochromatography
CE-MS	Capillary electrophoresis-mass spectrometry
CGE	Capillary gel electrophoresis
cIEF	Capillary isoelectric focusing
CZE	Capillary zone electrophoresis
EOF	Electro-osmotic flow
HILIC	Hydrophilic interaction liquid chromatography
ITP	Isotachophoresis
MEKC	Micellar electrokinetic chromatography
MSI	Multi-segment injection
NACE	Non-aqueous CE
SDS	Sodium dodecyl sulphate
TOF-MS	*Time-of-flight* mass spectrometry
μ_{ep}	Electrophoretic mobility

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1 Introduction

The aim of using metabolomics is to obtain insight into a well-defined biological question or problem [1]. For this purpose, targeted and untargeted metabolomics studies can be used. In the first approach, the focus is on the (quantitative) analysis of a set of well-defined metabolites or metabolite classes using a tailor-made sample preparation strategy, while in the second approach the focus is on analysing a broad range of metabolite classes without a priori knowledge of their nature or identity. The biological question often dictates whether a untargeted or targeted approach needs to be considered. Both approaches can be employed in a single metabolomics study, where the first approach is generally used to find potential biomarkers, and the second approach is then used to verify the results obtained with the first approach, preferably employing standardized protocols as required for biomedical and clinical studies. Ultimately, the use of metabolomics should provide an answer to the proposed biological question.

At present, the Human Metabolome Database is comprised of more than 115,000 metabolite entries, of which a major part consists of lipids and exogenous compounds derived from nutrients and drugs [2]. In order to probe as many (endogenous) metabolites as possible in a given biological sample, a combination of analytical techniques with complementary separation mechanisms is needed. For example, for the characterization of the human serum metabolome, multiple analytical separation techniques have been used in order to capture a broad range of endogenous metabolites [3]. In this case, more than 4000 metabolites were detected at concentrations spanning more than nine orders of magnitude.

Currently, nuclear magnetic resonance (NMR) spectroscopy, liquid chromatography (LC) and gas chromatography (GC) coupled to mass spectrometry (MS) are generally used for metabolomics studies [4–6]. Concerning chromatographic-based separation techniques, notably the use of LC columns based on sub-2 μm porous particles and/or core-shell silica particles has gained increased interest in metabolomics studies, as they can provide relatively fast separations with a high peak capacity [7]. In general, reversed-phase LC columns are employed for metabolomics, which can be used for the analysis of a wide range of metabolite classes. For the analysis of polar and charged metabolites, mainly ion-pair reversed-phase LC-MS and hydrophilic interaction liquid chromatography (HILIC) are considered. However, the use of ion-pair agents in LC-MS may result in severe ion suppression and may contaminate the ion source and ion optics [8]. Moreover, ion-pair agents may contribute to column instability and increased re-equilibration time. In HILIC a polar stationary phase is used in combination with aqueous organic eluents for the analysis of polar metabolites. This approach has gained interest as a complementary chromatographic separation technique for the profiling of polar metabolites over the past few years [9].

CE-MS is an analytical technique not commonly used in metabolomics, while it has very strong features for the analysis of highly polar and charged metabolites. Lack of (practical) expertise with this method and the perception that CE-MS is a technically challenging and not reproducible approach as compared to chromatographic-based methods may explain its limited use in metabolomics. However, a few recent studies clearly demonstrate the value of CE-MS for metabolic profiling of large sample sets [10, 11]. For example, the group of Soga and co-workers introduced the first CE-MS approaches for metabolomics in 2003 [12] and more recently has assessed the long-term performance of CE-MS for metabolic profiling of more than 8000 human plasma samples from the Tsuruoka Metabolomics Cohort Study over a 52-month period [11]. The study provided an absolute quantification for 94 polar metabolites in plasma with a similar or better reproducibility when compared to other analytical platforms.

In CE, compounds are separated on the basis of their intrinsic electrophoretic mobility, i.e. charge-to-size ratio. As such, the separation mechanism of CE is fundamentally different from chromatographic-based separation tech-

niques. Both CE and HILIC can be employed for the analysis of polar and charged metabolites; however, some crucial differences exist [13, 14]. For example, a relatively larger amount of the sample can be injected in HILIC-MS, which is an advantage when sample amount is not an issue. On the other hand, significantly higher separation efficiencies can be obtained by CE, and it is especially useful for the efficient analysis of low-abundance polar and charged metabolites in low sample amounts [15]. Moreover, analysis times are significantly shorter for CE because re-equilibration of the HILIC column is often a time-consuming process [5]. In addition, HILIC requires an important fraction of non-polar solvent in the sample, potentially leading to solvent incompatibility with the most polar compounds and reducing the polarity range of HILIC [16]. The clear complementary role of CE-MS in comparison to other analytical techniques for metabolomics studies has been recently demonstrated by various research groups [14, 17–21].

The development of new interfacing designs over the past few years resulted in a relatively increased interest of the CE-MS approach for metabolomics studies, but also in other fields. Moreover, progress has been made in improving the migration time repeatability and to further shorten the total analysis time. For a comprehensive overview of these developments in CE-MS for metabolomics, the reader is referred to the following reviews [22–28]. The aim of this chapter is to give an overview of the possibilities of CE-MS for metabolomics studies. Various CE separation modes employed for CE-MS-based metabolomics are discussed. Main aspects related to hyphenation of CE to MS are considered, and special attention is devoted to the use of new interfacing techniques including its implications for metabolomics studies. Subsequently, the utility of CE-MS for targeted and untargeted metabolomics is illustrated on the basis of the discussion of relevant biomedical and clinical examples. Finally, some general conclusions and perspectives are provided.

2 CE-MS Methodology

2.1 CE Systems for Metabolomics

CE has been used for the analysis of (endogenous) metabolites in various biological samples for more than a few decades now. Jellum et al. reported one of the first assays for the profiling of organic acids in human body fluids in order to screen for various metabolic diseases [29–31]. In one of the studies, about 50 metabolites could be observed in human urine within a short analysis time (<15 min) using minimal sample pretreatment [30]. Compound identification was performed by comparison of migration time and UV-visible diode-array spectra against known standards. The group of Barbas and co-workers also played an important role in the development of CE assays for the selective determination of organic acids in human urine, which were used for the diagnosis of inborn errors of metabolism [32].

Like in LC, CE can be used in a number of separation modes, such as capillary zone electrophoresis (CZE), referred to as "CE" in this chapter, micellar electrokinetic chromatography (MEKC), non-aqueous CE (NACE), capillary electrochromatography (CEC), capillary gel electrophoresis (CGE), capillary isotachophoresis (cITP) and capillary isoelectric focusing (cIEF). From the viewpoint of coupling CE to electrospray ionization (ESI) mass spectrometry, CE is used as the main separation mode as volatile buffers are required. For example, ammonium acetate, acetic acid and formic acid are frequently employed as background electrolyte (BGE) in CE-MS studies. The use of these BGE systems may not essentially provide the same level of separation performance as obtained with the typically employed phosphate- or borate-based BGEs in CE-UV mode. Though only a limited number of BGEs can be considered in CE-MS method development, the CE separation can be improved by adding organic modifiers to the BGE. For example, Mayboroda et al. improved the separation of leucine from isoleucine by using a BGE of 2 mol/L formic acid (pH 1.8) containing 20% (v/v) methanol [33].

In CE, compounds are separated according to differences in their intrinsic electrophoretic mobilities, which is dependent on the size (hydrodynamic radius) and charge of the ion, as well as the viscosity of the BGE. Under constant separation conditions, the electrophoretic separation of metabolites is solely based on differences in charge-to-size ratio. The use of high voltages leads to relatively fast separation times, as the migration time of compounds is inversely proportional to the electric field. Soga and coworkers developed the first CE-MS methods for untargeted metabolic profiling of biological samples [12, 34]. For the global analysis of cationic metabolites (basic compounds), a bare fused-silica capillary using 1 mol/L formic acid (pH 1.8) as BGE has been employed, while a cationic polymer-coated capillary using 50 mmol/L ammonium acetate (pH 8.5) as BGE has been employed for the global analysis of anionic metabolites (acidic compounds). In this latter method, neutral compounds are dragged to the detector with the electro-osmotic flow (EOF). However, as their charge is zero, they cannot be electrophoretically resolved. The use of both methods allowed the detection of more than 1600 molecular features in *Bacillus subtilis* extracts, of which 150 could be identified.

In MEKC, micelles are used as pseudo-stationary phases in the BGE allowing the concomitant separation of both charged and neutral analytes. However, the coupling of MEKC to MS is not straightforward due to the use of non-volatile micelles (often sodium dodecyl sulphate (SDS)). On the other hand, volatile surfactants may be considered to overcome these issues. For example, Moreno-González et al. developed a MEKC-MS method for the analysis of amino acids in human urine using ammonium perfluorooctanoate (APFO) as a volatile surfactant [35].

In CEC, separation of compounds is based on differences in electrophoretic mobility and the partition between a stationary and mobile phase. The mobile phase flows through the column by the EOF, generated by the silanol groups of the stationary phase. Neutral compounds move through the packed column by the EOF and are separated by partitioning between the stationary and mobile phase, whereas charged compounds move through the packed column with the additional contribution from the intrinsic electrophoretic mobility of the analytes. Given the separation mechanism of CEC, this CE mode is useful for the efficient separation of a wide range of compounds. Recently, Wu et al. reported the development of a pressurized CEC method hyphenated to MS for profiling metabolites in human urine [36]. A home-made sheathless interface was constructed for hyphenating CEC to MS in order to take advantage from the intrinsically low flow rate of pressurized CEC. The analytical performance of the CEC-MS method was evaluated with a metabolite test mixture and pooled human urine**.** Limits of detection (LODs) for test compounds ranged from 18 to 88 ng/mL. Multivariate data analysis of urine metabolite profiles distinguished lung cancer patients from controls. For metabolite identification, the selectivity provided by CEC was critical in order to distinguish fragment ions of glutamine conjugates from co-eluting metabolites. Three glutamine conjugates, i.e. phenylacetylglutamine, acylglutamine C8:1 and acylglutamine C6:1, were identified among 16 distinct metabolites in this study. The utility of CEC-MS for metabolomics studies has hardly been explored so far. A potential reason for that may be related to the design of consistent CEC columns.

The other CE separation modes, i.e. CGE and cIEF, are generally used for protein analysis. Still, cIEF may have potential for metabolomics studies as it is very suited for the high-resolution separation of amphoteric compounds according to their isoelectric point (pI). In cIEF, a mixture of ampholytes and sample fills the capillary. A basic catholyte, generally sodium hydroxide, is placed at the cathode, and an acidic anolyte, e.g. phosphoric acid, is placed at the anode. Due to the ampholyte properties, a pH gradient is established in the capillary under the influence of an electric field. Amphoteric compounds are focused until the region where the pH and the pI of the amphoteric compounds are equal; thus, the net charge of the compound is zero, and, as a result, the compound will not migrate. Electrophoretic or hydrodynamic mobilization is then used to

move the individual components to the detector [37].

When employing CE systems based on bare fused-silica capillaries, variability of migration times due to adsorption of sample matrix components to the inner capillary wall may become an issue, especially when the purpose is to analyse large sample cohorts by CE-MS. Obviously, migration time variability should be very low for comparative metabolic profiling studies. A way to minimize this is by using coated capillaries. For an overview of the use of coated capillaries in CE-MS and more specifically for CE-MS-based metabolomics studies, we refer to the following papers [38, 39].

2.2 Interfacing Designs and Their Implications for Metabolomics

For metabolomics studies, MS is an indispensible analytical tool, especially from the viewpoint of the reliable identification of compounds present in a given biological sample. For untargeted metabolomics, *time-of-flight* MS (TOF-MS) instruments are generally employed for obtaining full-scan MS recordings with a high mass accuracy. Moreover, due to its high spectral acquisition rate, TOF-MS instruments are fully compatible with highly efficient CE separations (i.e. very sharp peaks are generated). For targeted metabolomics, TOF-MS, ion trap MS and triple quadrupole MS systems are generally employed in combination with CE [5, 40–43].

For the coupling of CE to MS, a special interface is required in order to perform the electrophoretic separation independent from ESI-MS, i.e. the electric fields used for both processes should not interfere with each other. Such an interfacing design for coupling CE to MS was first developed by Smith and co-workers, in which configuration a co-axial solvent (i.e. sheath-liquid) was delivered as a terminal electrolyte reservoir [44]. This interfacing design was further improved and commercially available since 1995 as a co-axial sheath-liquid interface. In this design, the CE capillary is inserted into a larger diameter tube (Fig. 1). The sheath-liquid, to which the CE terminating voltage is applied, is provided via an outside tube and merges with the CE effluent at the capillary outlet. A gas flow is often applied via a third co-axial capillary to facilitate spray formation in the ESI source. In principle, the flow rate and composition of the sheath-liquid dictate the ESI process in this particular configuration, and, as such, the selection and optimization of the sheath-liquid is very critical. In general, the sheath-liquid is composed of a mixture of water and organic modifier, including a small percentage of a volatile acid or base (e.g. formic acid or ammonium acetate), and provided at flow rates typically between 2 and 10 μL/min.

Until now, the co-axial sheath-liquid interface (or conventional interface) has been most often used for coupling CE to MS in a wide range of application fields [45–48]. Although this interfacing design can be used in a rather robust way [49], the CE effluent is significantly diluted, thereby often resulting in low- to mid-μmol/L detection limits for metabolites when using standard injection volumes [50, 51]. In-capillary preconcentration techniques, such as transient isotachophoresis or dynamic pH junction, can be used to further improve the detection sensitivity of sheath-liquid CE-MS for metabolomics studies [52, 53]. Another way to enhance detection sensitivity is by adding modifiers to the sheath-liquid [54]. For example, enhanced charging or supercharging of compounds in CE-MS has been explored for the analysis of intact proteins by the addition of various supercharging agents, such as 3-nitrobenzyl alcohol and sulfolane, to the sheath-liquid in order to modulate the charge-state distribution of proteins [55]. The potential of using this strategy in CE-MS-based metabolomics studies has not been explored so far.

Over the last decade, modified versions of the conventional co-axial sheath-liquid interface have been developed in which the sheath-liquid is provided at a significantly lower flow rate [56]. In some cases, such designs have been designated as a liquid junction interface; however, in our opinion, they can be considered as low-flow or miniaturized sheath-liquid interfaces. Maxwell et al. developed such a low-flow interface by using a flow-through micro-vial interface, which was

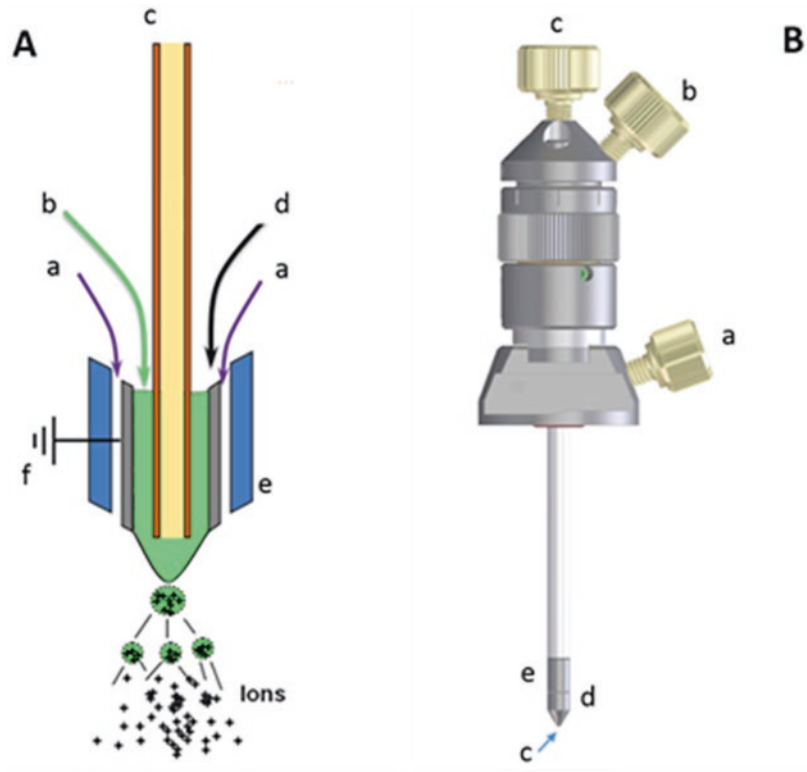

Fig. 1 Graphic representation of an Agilent co-axial sheath-liquid CE-MS interface: (a) nebulizing gas, (b) sheath-liquid, (c) CE capillary with BGE, (d) stainless steel spray needle 0.4 mm i.d., 0.5 mm o.d., (e) outer tube and (f) ground connection. (**b**) Engineering sketch of the co-axial sheath-liquid CE-MS interface. (Reproduced from reference [56] with permission)

created by inserting the separation capillary into a tapered stainless steel hollow electrospray emitter [57]. The small volume between the capillary and inner walls of the needle electrode tip formed a flow-through micro-vial that acted as both the outlet vial and the terminal electrode (Fig. 2). The flow-through micro-vial also allowed the addition of a sheath-liquid solution at low flow rates to provide a stable flow to the needle tip, which increased the compatibility of the CE effluent with ESI while minimizing sample dilution. The bevel on the emitter tip resulted in increased spray stability and effectively moved the ionization site away from the outlet.

The analytical performance of CE-MS using the flow-through micro-vial interface for metabolomics was recently assessed by Lindenburg et al. [58]. Using a standard metabolite mixture, the flow-through micro-vial (500 nL/min flow rate) and the standard sheath-liquid CE-MS interfaces (4 μL/min flow rate) were compared (Fig. 3). The LODs obtained with the flow-through micro-vial interface ranged from 0.01 to 3 μmol/L, which was on average a fivefold improvement as compared to the LODs obtained by conventional CE-MS. The potential of CE-MS using the flow-through micro-vial interface utility for metabolic profiling of large sample sets has not been shown so far. A drawback of this interface is the low tolerance to high CE currents as formation of electrolysis gases may accumulate in the spray needle and, as a result, hindering electrical contact. A nice feature of this approach is that it allows the use of CE capillaries with different inner diameters.

Next to the sheath-liquid interfaces, sheathless interfaces can be employed for coupling CE to MS. In such designs the CE voltage is applied directly to the BGE at the capillary outlet [59]. For conductivity, the metal may be coated on the end of the tapered separation capillary. Alternatively, a metal-coated, full metal or conductive polymeric sprayer tip may be connected to the CE outlet [56]. A closed electrical circuit may also be created by inserting a metal microelectrode through the capillary wall into the

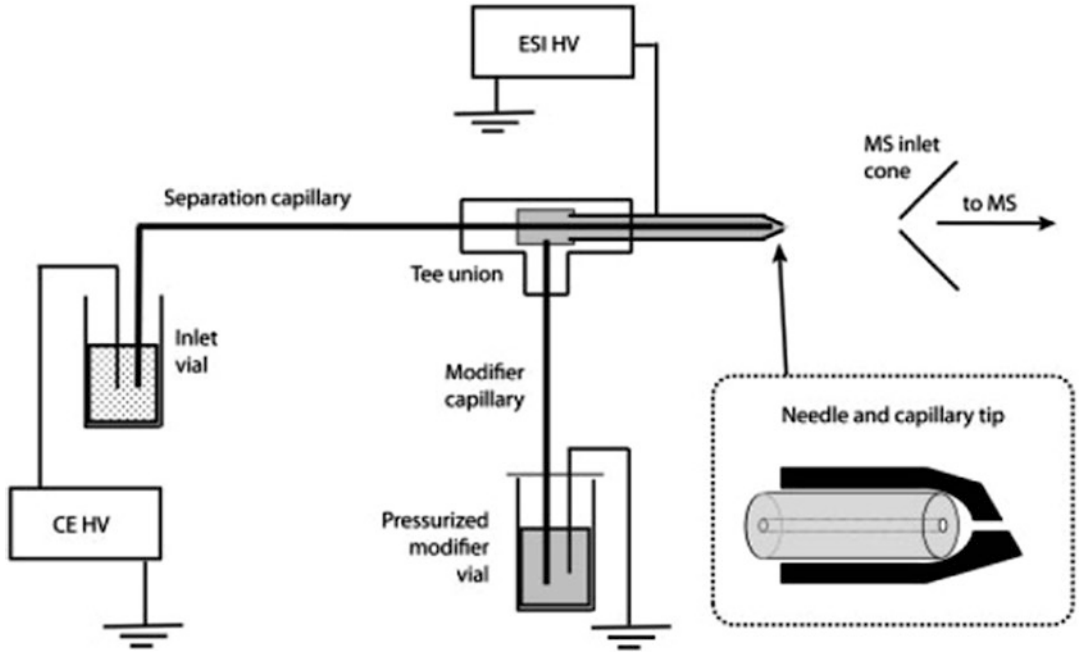

Fig. 2 Schematic illustration of the flow-through micro-vial interface apparatus, including a dissected view of the needle tip with inserted capillary (inset). (Reproduced from reference [57] with permission)

BGE, by direct introduction of a micro-electrode into the capillary [60, 61] or by a crack in the silica wall close to the capillary tip [62].

A CE-MS method using a sheathless porous tip interface, which was first developed by Moini [63], has been developed for the global profiling of cationic metabolites in human urine [53]. The porous tip interface was designed by removing the polyimide coating of the capillary outlet and etching the capillary wall with 49% solution of hydrofluoric acid to a thickness of about 5 μm (Fig. 4). The electrical connection to the capillary outlet was obtained by inserting the etched conductor into an ESI needle, which was filled with BGE. The sheathless porous tip interface was mainly useful for interfacing narrow capillaries (<30 μm inner diameter) and for low-flow-rate (<100 nL/min) nano-ESI-MS analyses. Using human urine, this approach allowed obtaining a highly information-rich metabolic profile as compared to CE-MS employing a sheath-liquid interface. Around 900 molecular features were observed with sheathless CE-MS, while only 300 were detected by sheath-liquid CE-MS. Overall, the improved detection sensitivity of sheathless CE-MS significantly improved the coverage of the urinary metabolome. However, with a single porous tip capillary emitter around 100 pretreated biological samples can be analysed at this stage, which is not really cost-effective given the relatively high price of a single porous tip capillary.

Apart from body fluids, the utility of CE-MS using a sheathless porous tip interface has also been recently evaluated for metabolic profiling of low number of mammalian cells, using HepG2 cells as a model system [64]. Given the nanomolar concentration sensitivity, the sheathless CE-MS method could be effectively used for obtaining metabolic profiles in HepG2 cells starting from 10,000 down to 500 cells. A typical profile obtained for cationic metabolites from a starting amount of 500 HepG2 cells only is shown in Fig. 5, in which more than 20 metabolites could be observed. Hence, these results suggest that the method has the sensitivity for performing single-cell mammalian metabolomics studies. Still, the long-term performance of

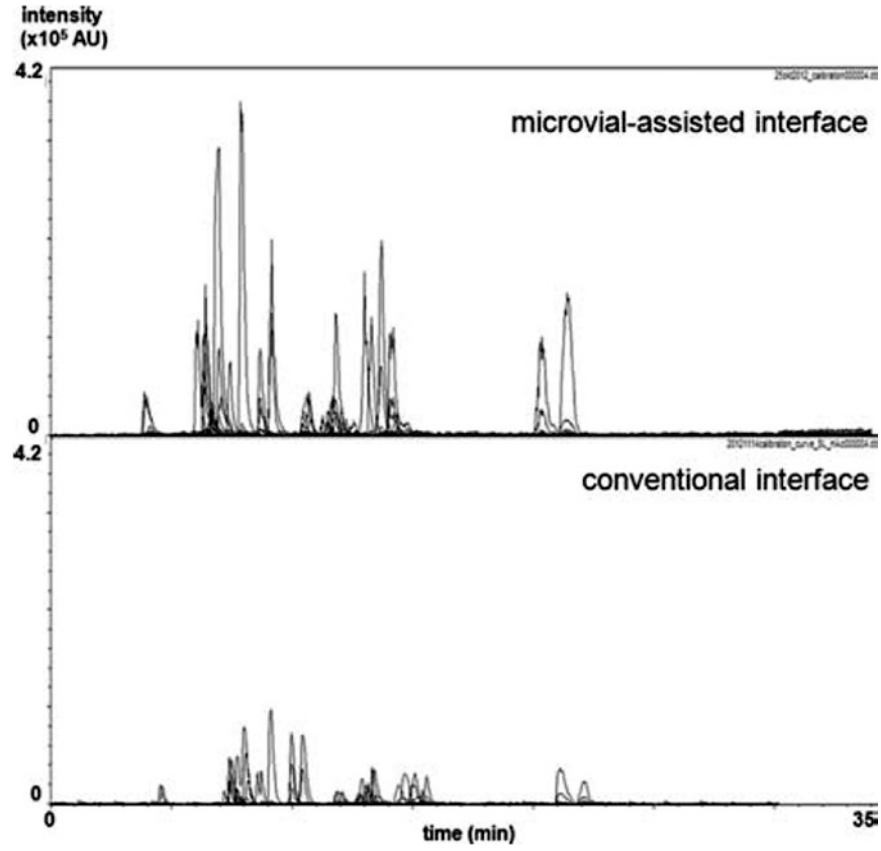

Fig. 3 Multiple extracted ion electropherograms of 35 cationic metabolites (25 μmol/L) obtained by CE-MS using a flow-through micro-vial interface (upper electropherogram) and a sheath-liquid interface (lower electropherogram). (Reproduced from reference [58] with permission)

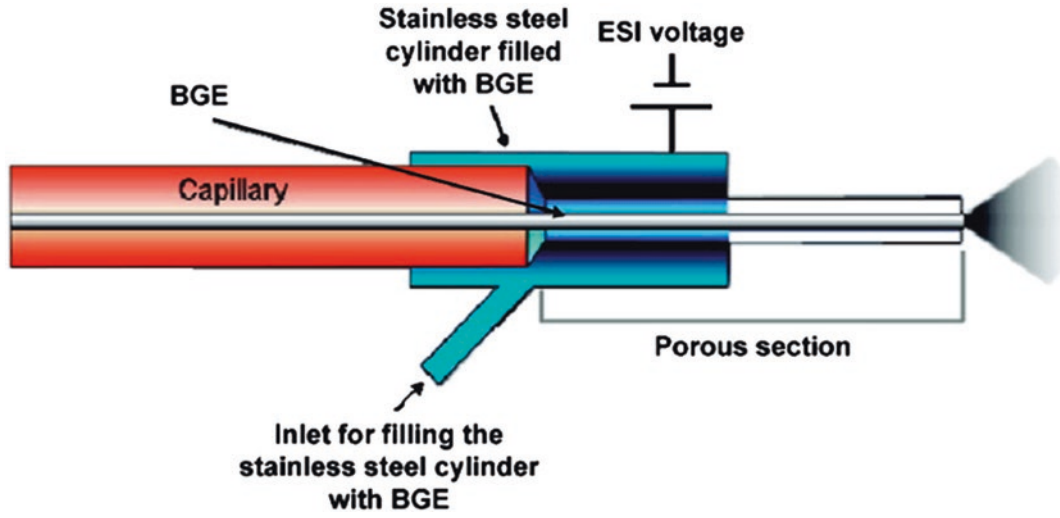

Fig. 4 Schematic of the high-sensitivity porous sprayer sheathless interface. (Reproduced from reference [59] with permission)

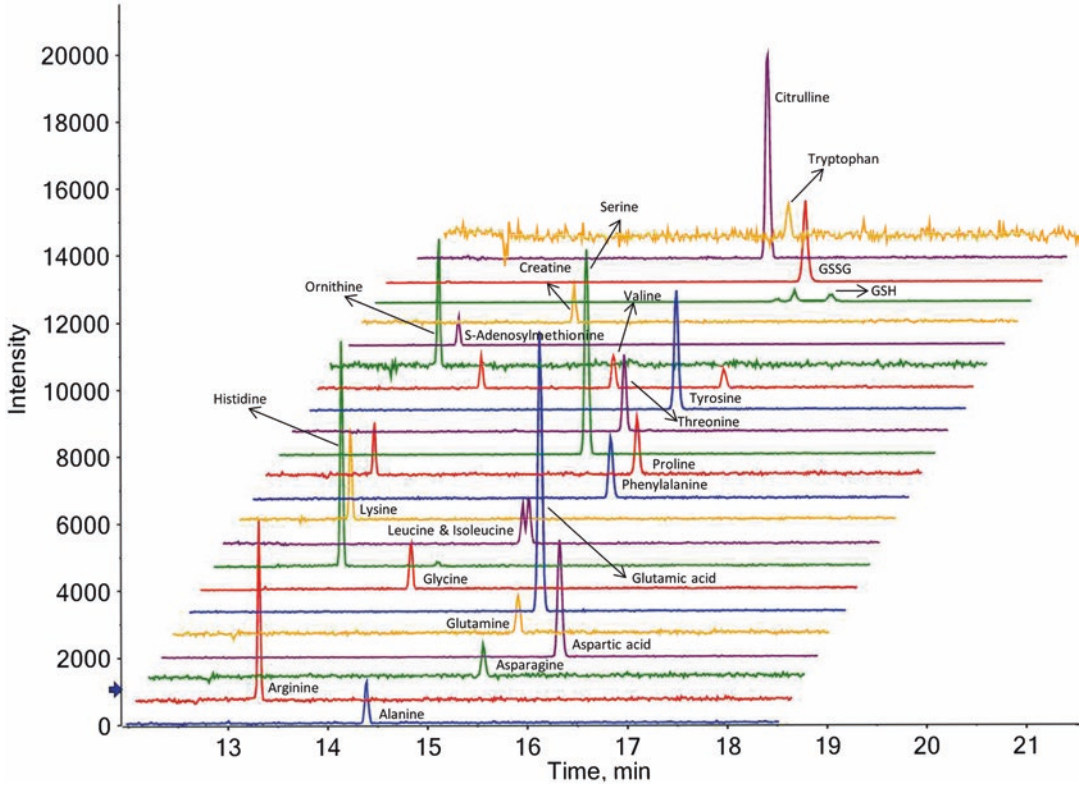

Fig. 5 Multiple extracted ion electropherograms for a selected number of metabolite peaks detected in an extract of 500 HepG2 cells by sheathless CE-MS in positive mode using a porous tip emitter. Separation conditions: BGE, 10% acetic acid (pH 2.2). Separation voltage: 30 kV. Sample injection: 6.0 psi for 60 s. (Reproduced from reference [64] with permission)

this approach needs to be assessed more comprehensively using substantially larger numbers of clinical samples.

3 Applications

In metabolomics, two different analytical approaches are typically used, targeted and untargeted analysis. Targeted metabolomics focuses on the quantification of a limited number of analytes. Therefore, accent is made on analysis throughput, sensitivity and robustness of the methods. In contrast, untargeted metabolomics focuses on every detectable feature in order to obtain a (unique) chemical fingerprint of the sample. Therefore, focus is made on unambiguous identification of the detected features. In the following sections, some state-of-the-art targeted and untargeted metabolomics studies performed by CE-MS are highlighted.

3.1 Targeted Applications

Nowadays, single-cell analysis remains a major challenge for current analytical platforms due to the large dilution required to handle the content of single cell, but also to selectively extract the relevant fraction for follow-up analysis. This kind of analysis is made even more complicated when the focus is on phosphorylated compounds, which are difficult to analyse by reversed-phase LC approaches due to their very high polarity. In this context, Liu et al. developed a CE-MS method for the quantification of 16 nucleotides

from R2 neurons of *Aplysia* central nervous system (CNS) (300 µm diameter) [65]. To do this, they employed a home-made nano-flow sheath-liquid interface operating at 600 nL/min in ESI negative mode. After optimization of the BGE (20 mmol/L ammonium bicarbonate) and sheath-liquid composition (50% isopropanol containing 0.2 mol/L BGE), they were able to measure sub-picomole levels of nucleotides in a single cell (Fig. 6). To adapt their method to smaller cells such as *Aplysia* sensory neurons (i.e. 60 µm diameter), they successfully employed a large volume sample stacking procedure, leading to a sensitivity increase of up to 200-fold resulting in a detection of 51 fg of material.

Chiral amino acids are known for their importance in many biological processes, including pathologies such as schizophrenia, ischemia, epilepsy and neurodegenerative disorders [66]. For this reason, chiral analysis of the metabolome is becoming an important field of research. CE is very powerful for chiral separations when chiral selectors such as cyclodextrins [67] or crown ether carboxylic acids [68] are added to the BGE. However, these selectors are not MS compatible, leading to the necessity to develop alternative strategies. Recently, Sánchez-López et al. used a partial filing injection approach in order to separate enantiomers of underivatized amino acids and derivatives from the phenylalanine-tyrosine pathway [67]. To do this, they optimized the BGE composition which was made of a mixture of hydroxypropyl- and methyl-β-cyclodextrins (40 mmol/L and 180 mmol/L, respectively, in 2 mol/L formic acid). In order to circumvent the deleterious effect of suction effect on chiral separation, they had to use a 120 cm total capillary length, leading to very long analysis times. The developed method was also compatible with large volume stacking (7% of the capillary length) without compromising the resolution, leading to a sensitivity increase of up to 50-fold (Fig. 7).

CE-MS may be considered a relatively slow method if long capillary and extensive rinsing procedures between sample injections are used, making the total analysis about 30 min. To reduce the total analysis time required per sample, injection of multiple samples in a single analysis was developed more than a decade ago by Geiser et al. [69, 70]. This concept was re-discovered and further optimized by Kuehnbaum et al. [71] and is now known as multi-segment injection (MSI). MSI allows to inject up to 7 or more discrete sample zones in a single electrophoretic run, thereby increasing the throughput to only a

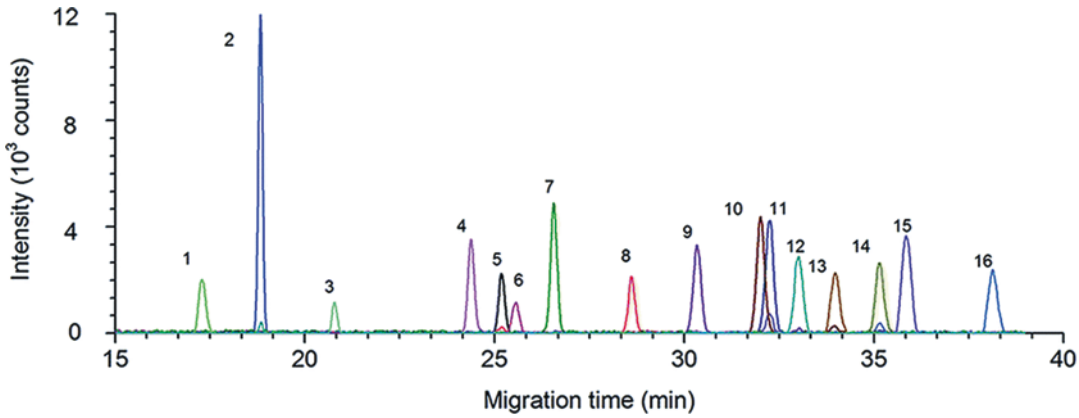

Fig. 6 Extracted ion electropherograms acquired from solutions containing 16 anionic analyte standards by CE-MS. Injection volume, 10 nL; BGE, 20 mmol/L ammonium bicarbonate (pH 10); separation voltage, 10 kV. Analysed compounds (each at 100 µg/L): 1 – NAD+ (*m/z* 622.102); 2 – cAMP (*m/z* 328.045); 3 – FAD (*m/z* 784.150); 4 – AMP (*m/z* 346.056); 5 – CMP (*m/z* 322.045); 6 – NADP+ (*m/z* 742.068); 7 – GMP (*m/z* 362.058); 8 – UMP (*m/z* 323.029); 9 – ADP (*m/z* 426.022); 10 – GDP (*m/z* 442.017); 11 – CDP (*m/z* 402.011); 12 – ATP (*m/z* 505.989); 13 – GTP (*m/z* 521.983); 14 – UDP (*m/z* 402.995); 15 – CTP (*m/z* 481.977); and 16 – UTP (*m/z* 482.961). (Reproduced from reference [65] with permission)

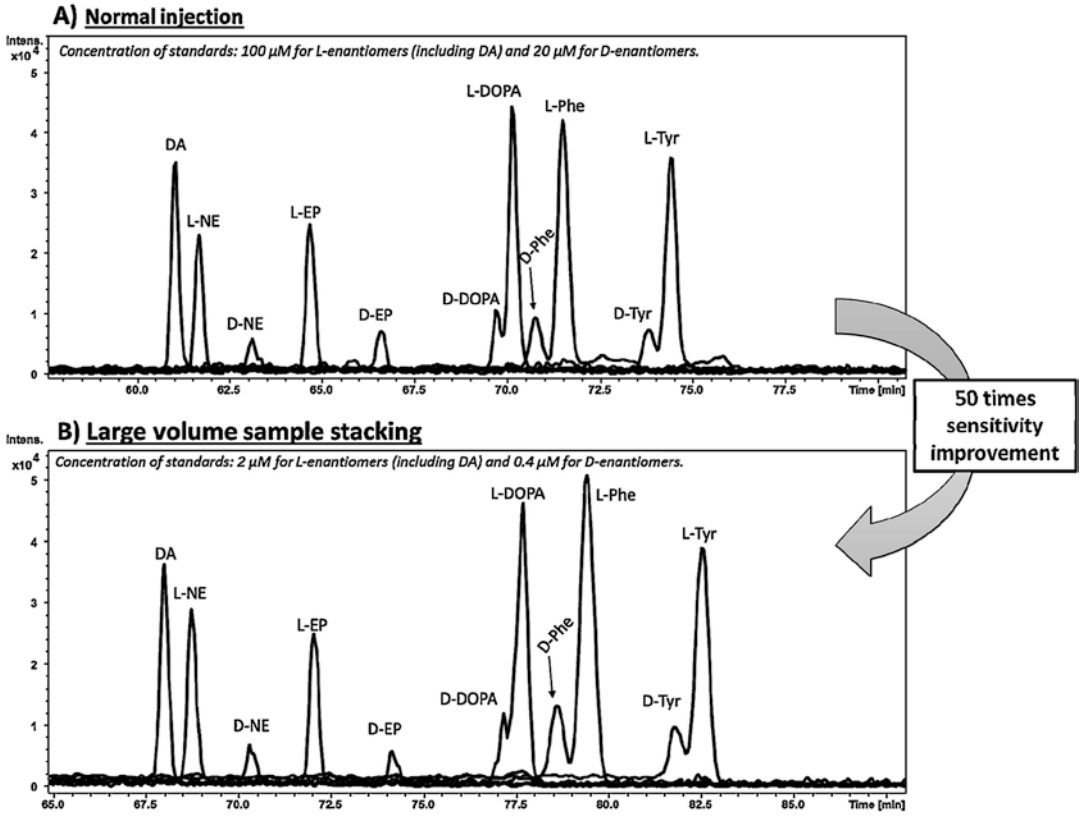

Fig. 7 Extracted ion electropherograms obtained by CE-MS for the chiral separation of the Phe-Tyr metabolic pathway constituents with injection time of 5 s (normal injection) in (**a**) and 250 s (injection with large volume sample stacking, LVSS) in (**b**). Peak identification: dopamine (DA), norepinephrine (NE), epinephrine (EP), 3,4-dihydroxyphenylalanine (DOPA), phenylalanine (Phe) and tyrosine (Tyr). (Reproduced from reference [67] with permission)

few minutes per sample. This method was successfully applied to various clinical studies, i.e. from the monitoring of 52 drugs of abuse [72] in human urine to the monitoring of inborn errors of metabolism from dried blood spot samples [73]. More recently, MSI was used in NACE-MS for the analysis of 18 fatty acids from C10 to C24 [74]. To reach a good separation of the metabolites, a complex BGE made of 35 mmol/L ammonium acetate in 70% v/v acetonitrile, 15% v/v methanol, 10% water and 5% v/v isopropanol with an apparent pH of 9.5 was used (Fig. 8). The developed NACE-MS method yielded a linear dynamic of at least two orders of magnitude for the tested compounds, with an average limit of quantification (LOQ) of 2.4 µmol/L. A further validation was performed by comparison of the newly developed MSI-NACE-MS with a standard GC-MS method, which revealed a similar sensitivity of both approaches; however, MSI-NACE-MS lacked isomeric resolution for some minor fatty acids.

As an alternative to using hydrodynamic separation plugs between the samples in MSI CE-MS, Drouin et al. proposed the use of electrokinetic plug to separate the samples [21]. This approach has the main advantage to circumvent the deleterious effect of multiple pressure applications, which can lead to peak diffusion and, as a result, to a loss of separation efficiency. Moreover, this approach also provides a longer effective capillary length for separation. With electrokinetic

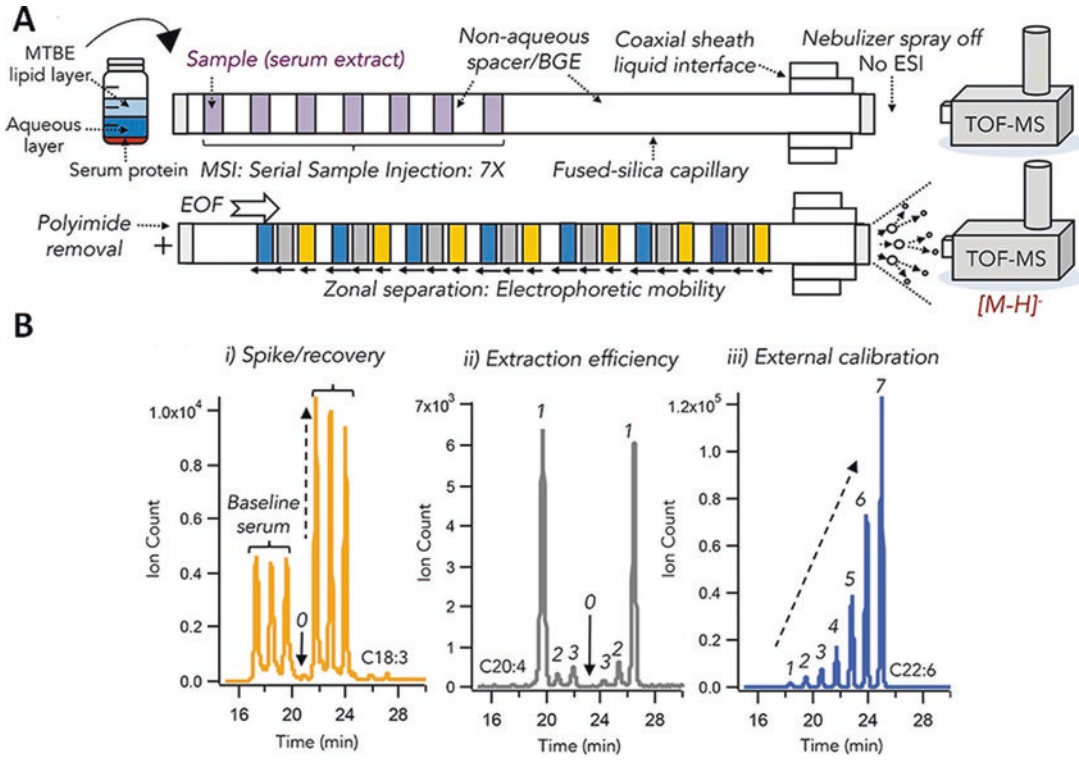

Fig. 8 (**a**) Multiplexed separation of fatty acids by NACE-MS using serial injection of seven or more discrete sample segments and their zonal electrophoretic separation following MTBE serum extraction with full-scan data acquisition under negative ion mode detection. (**b**) Customized serial injection configurations used in MSI-NACE-MS for FA quantification, including spike/recovery study for determination of method accuracy (in triplicate) and repeated MTBE serum extracts to evaluate extraction efficiency (in duplicate) along with seven-point external calibration curve over a 200-fold concentration range (1–200 μM). (Adapted from reference [74] with permission)

injection, optimization of space length and number of injected samples is simplified. However, the number of samples is limited by the migration time of the fastest compounds and the duration of the plugs.

Recently, Ouyang et al. developed a cIEF-MS method in order to achieve the separation of acidic oligosaccharides [75]. Using a set of commercial ampholytes with a pH range from 2.5 to 5.0, they were able to separate a mix of 16 disaccharides from different families. To do this, a reversed separation polarity was employed (Fig. 9). Under these conditions, the anolyte solution (0.1–1% formic acid) was injected first, followed by the sample mixed with the ampholytes, and then the catholyte solution (0.2 mol/L ammonium hydroxide) was injected. Therefore, when a negative voltage of −30 kV was applied, a gradient pH was generated (from 5 at the inlet to 2.5 at the outlet) and compounds of interest migrated and were stacked in the zone where the pH was equal to their pI. Despite the presence of methanol in the solution, the gradient of apparent pH formed was demonstrated as linear based on the used pI markers. This method showed good repeatability as well as a good separation power for isomeric compounds. Indeed, it was possible to separate O- and N-sulfo isomers based on their slight pI difference. Finally, this method was successfully used for the separation of larger chondroitin sulphate oligosaccharides (tetra- and hexa-saccharides).

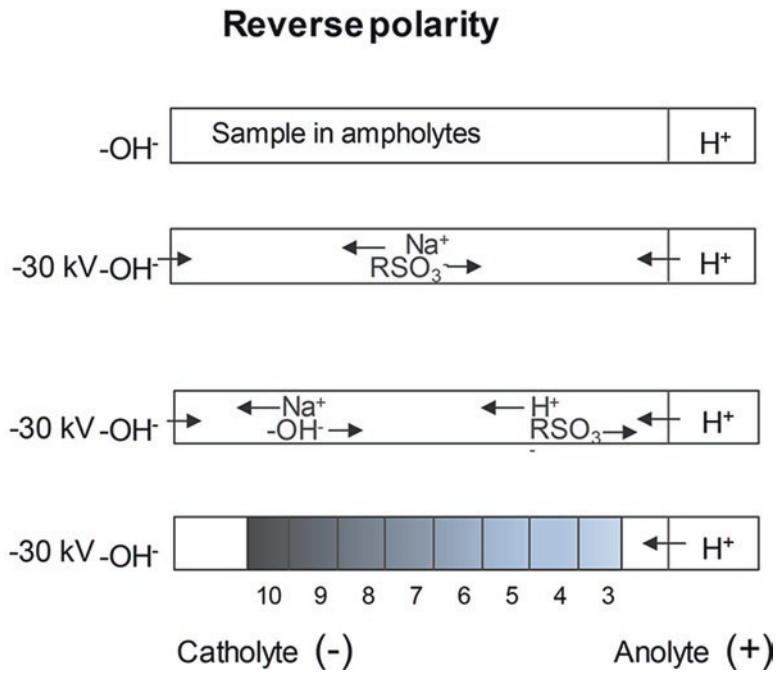

Fig. 9 Schematic of negative-ion mode cIEF-MS workflow showing reverse polarity separations. Anolyte solution (0.1–1% formic acid) was injected first, followed by sample mixed with the ampholytes (pH 2.5 to 5.0), and then catholyte solution (0.2 mol/L ammonium hydroxide) was injected. (Adapted from reference [75] with permission)

3.2 Untargeted Applications

Due to the lack of robustness and sensitivity often observed in CE-MS, LC-MS-based methods are generally used for untargeted metabolomics studies. However, recent developments in CE-MS indicate that this approach can reach the level of performance required for metabolomics. For example, in terms of robustness, a study involving the analysis of more than 8400 participant plasma samples was recently conducted by Harada et al. [11]. In this work, only 0.5% of the runs failed due to capillary current issues, highlighting the robustness of CE-MS.

In case of very limited sample amounts, such as single-cell profiling, CE-MS is extremely useful. Indeed, the extremely small injection volume limits the dilution factor required for injection and the nanospray in nano-sheath flow or in sheathless interfaces, leading to high detection sensitivities. In a recent work, Portero and Nemes were able to profile both anionic and the cationic metabolites of the left animal-ventral cell from a *Xenopus laevis*'s eight-cell embryo (Fig. 10) [76]. To do this, they designed a CE system which was capable to handle sample volumes below 1 μL. In order to avoid electrical discharge in ESI negative mode, the environment of the nanospray interface was saturated with acetonitrile gas. The LOD of the developed CE-MS method was on average around 5 nmol/L (~50 amol) for the tested compounds.

In another study, Sanchez-Lopez et al. took advantage of the CE-MS characteristics for profiling cationic metabolites in renal biopsies (20 μm thickness) using a sheathless porous tip interface [77]. However, in this study, only 5 metabolites were unambiguously identified using authentic standards and 21 compounds were putatively identified based on their accurate mass and MS/MS spectra. Indeed, one of the main challenges in untargeted metabolomics remains the annotation of the compounds. Criteria to achieve unambiguous identification (level 1) are extremely strict and require at least two independent and orthogonal parameters of a reference compound analysed under identical experimental conditions in the same laboratory [78–80]. Therefore, identification based on MS and MS/MS spectra search in libraries such as Metlin or HMDB can only reach the identification level 2 [2, 81]. In LC-MS-based methods, accurate mass

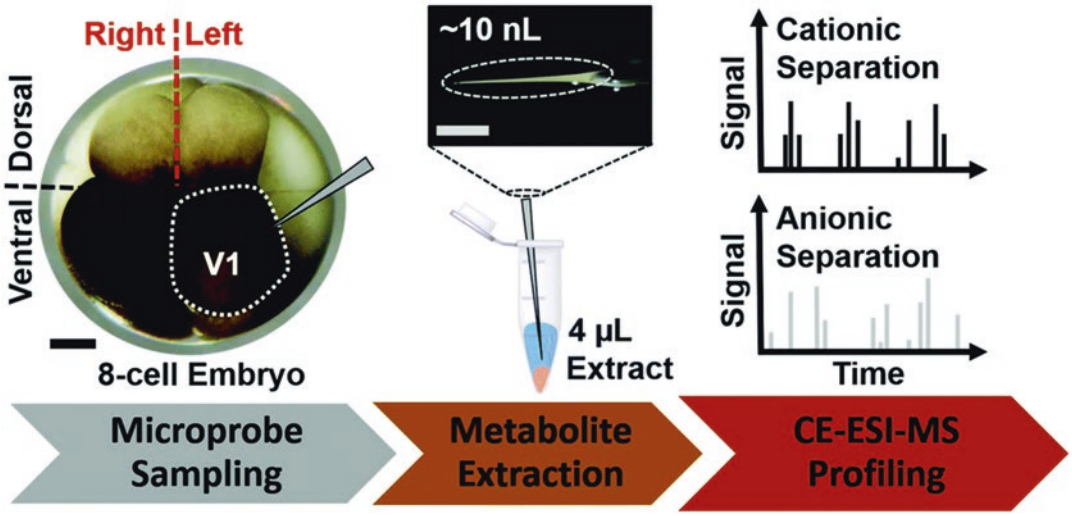

Fig. 10 Microprobe CE-MS strategy to measure cationic and anionic metabolites from the same identified cell in a live *X. laevis* embryo. The left animal-ventral (V1) cell of the eight-cell embryo was identified, and ~10 nL of its content was aspirated for one-pot metabolite extraction, followed by cationic and anionic profiling of the same cell extract. Scale bars = 250 µm. (Reproduced from reference [76] with permission)

and retention are commonly used for this purpose. However, the high variability of the migration times makes this strategy not very suitable in CE-MS-based analysis. To circumvent this problem, experimental relative migration times are commonly used [82, 83]. However, due to the cumulative effect of electrophoretic mobility and EOF on migration time and consequently on relative migration time, this approach is biased.

In comparison to migration time, under given conditions of BGE (pH and ionic strength) and temperature, electrophoretic mobility (μ_{ep}) can be considered as a physicochemical property of every compound (Eq. 1).

$$\mu_{ep} = \frac{z}{4.\pi.\eta.r} \quad (1)$$

where z is the charge of the molecule, η is the viscosity of the BGE and r is the hydrodynamic radius of the compounds. Therefore, μ_{ep} can be used for feature annotation. For this reason, Drouin et al. have created an experimental μ_{ep}-database [21]. To do this they used a two-step approach, with positive and negative CE polarity applied for cationic and anionic profiling, respectively. In both cases, a positive pressure was applied at the inlet of the capillary in order to reduce analysis time and generate an anodic flow in reverse CE polarity mode. Therefore, this approach allows global metabolic profiling of the sample in 20 min while maintaining a good peak shape and good separation of isomeric compounds, such as citric and isocitric acid (Fig. 11). In this study, 10% acetic acid buffer was used as BGE for both cationic and anionic metabolic profiling. Despite its low pH, this BGE was already reported earlier for cationic compounds as well as anionic species [84] and have as main advantages to be very easy and repeatable to prepare BGE. This method allows the detection of more than 450 compounds over a large variety of biochemical families (Fig. 12). To calculate the μ_{ep} of every compound, paracetamol was used as a neutral marker and was spiked in every sample. As expected, these strongly acidic conditions are highly suitable for basic compounds; however, as shown in Fig. 12, a large part of the acidic compounds are slightly charged and present a low μ_{ep} which can be detrimental for feature annotation. However, neutral compounds with the same exact mass, such as carbohydrates, cannot be separated or unambiguously identified with this method.

Interestingly, ammonium ions present in the sheath-liquid allowed the detection of a large

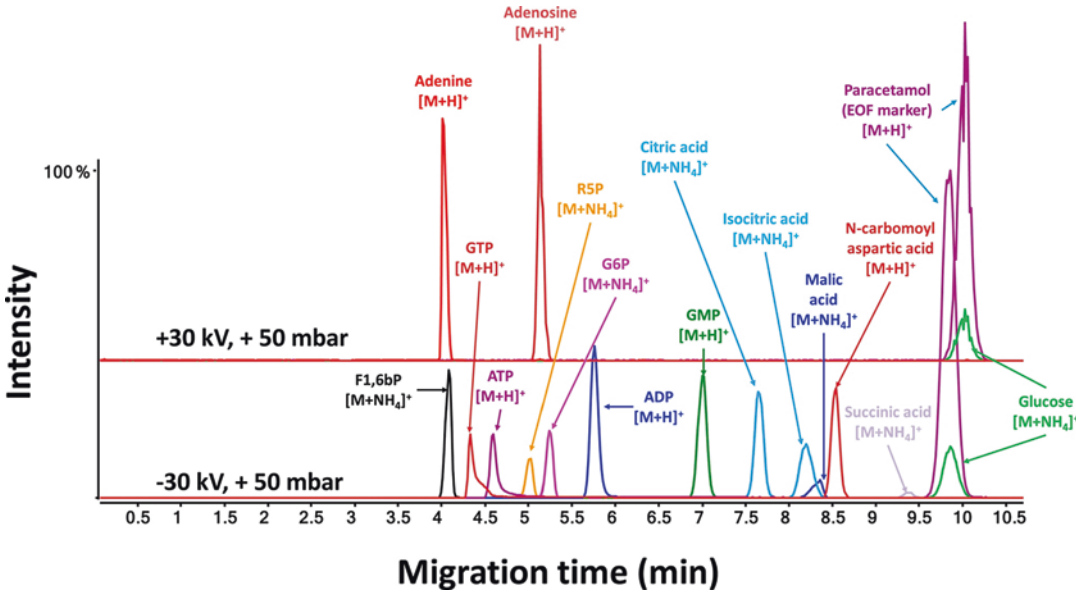

Fig. 11 Analytes observed in a metabolite test mixture by CE-MS in positive ESI mode using no nebulizer gas and an increased ESI voltage (5.5 kV). CE in normal polarity was used for cationic metabolites, while for anionic metabolites the polarity was reversed. (Reproduced from reference [21] with permission)

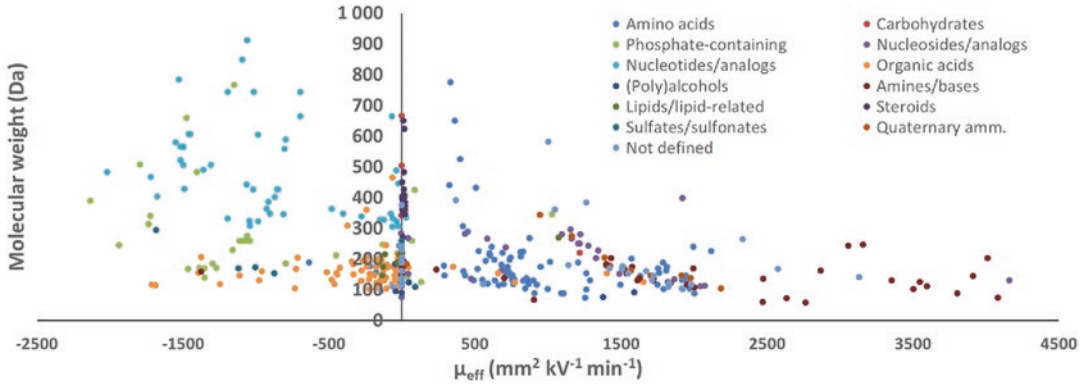

Fig. 12 Plot showing the detected compounds in a metabolite test mixture according to their effective mobilities and molecular weights. (Reproduced from Ref. [21] with permission)

variety of acidic metabolites in ESI positive mode by their ammonia adducts. However, the presence of ammonia in the sheath-liquid can also be considered as a drawback of this approach. Indeed, due to the reverse CE polarity applied during anionic metabolic profiling, ammonium ions, which have a very high but opposite mobility, migrate into the capillary, leading to the generation of a dynamic pH gradient in the capillary. In addition, the rising speed could be different between CE-MS platforms, generating a shift of the measured μ_{ep} from one CE-MS platform to another. However, for compounds with a sufficient μ_{ep}, this drawback was circumvented with fine-tuning of the positive pressure applied during the negative profiling, making the library transferable from one laboratory to another. Finally, after creation of the database in a first laboratory, it was successfully used for compound identification from cell culture extracts in a second laboratory, and 77 features were successfully annotated, with relative error to the

database below 5% for 90% of the identified metabolites.

In order to support this new methodology and ease its integration in conventional data treatment workflow, Gonzàlez-Ruiz et al. have developed a software designated as ROMANCE (RObust Metabolomic Analysis with Normalized CE) [85]. This open-access software performs a point-to-point conversion of the migration time scale into a μ_{ep} scale from *mzml* files. Due to high reproducibility of the electrophoretic mobilities, another advantage of the point-to-point conversion of the scale is to induce a peak alignment of the detected peak based (Fig. 13). However, this new concept suffers from a few drawbacks: (i) a new database must be created for every different BGE conditions, (ii) new conditions should be dedicated to acidic compound profiling to improve robustness of the method and separation of acidic compounds, and (iii) this approach is only possible with robust and easy to prepare BGE systems. In addition, the universal aspect of this approach must be demonstrated through a large inter-laboratory study.

A typical untargeted metabolomics analysis typically contains more than 10,000 features. A large part of them do not arise from the sample matrix but are just instrumental noise. Therefore, isolation of the relevant feature is an important step of data treatment. To do this, several approaches exist. One of them consists of the use of the injection of several dilutions of a quality control sample. Therefore, only the features following the dilution trend are considered for further data treatment [86, 87]. Through the MSI approach, this concept was extended to a dilution pattern of specimen samples [88]. In brief, an MSI of three pairs of samples at different dilution factor (1:2; 1:1, 2:1) is injected with a QC sample. Thereby, only the feature following this highly specific pattern is considered. Another advantage of MSI in untargeted analysis is the accurate correction of the analytical drift with injection of QC sample on every analysis. However, attribution of features to a specific injection and their annotation is made extremely difficult in the case of metabolites which are detected near their LODs in the samples. In addition, this step can only be performed manually, making the data pre-treatment procedure a time-consuming exercise. Indeed, these steps may only be realized if the complete MSI profile is obtained for each of the injections.

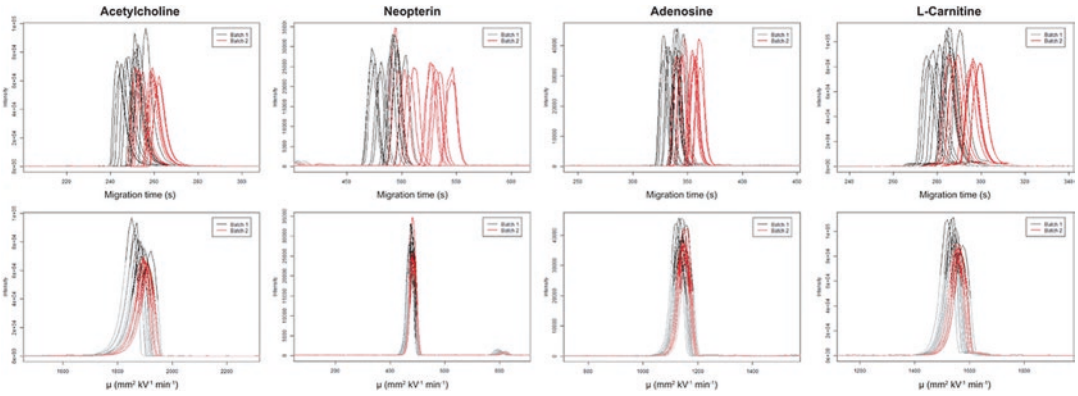

Fig. 13 Effect of the transformation into μ_{ep}-scale on a set of replicate analyses of a mix of standard compounds (n = 12), using two different batches of BGE (j = 2, red and black colours). The achieved pseudo-alignment is evident when top and bottom figures are compared. The upper electropherograms show the migration times of the compounds, while the lower electropherograms show the electrophoretic mobilities of the same compounds. (Reproduced from Ref. [85] with permission)

4 Conclusions and Perspectives

Over the past few decades, CE-MS has emerged as a strong analytical technique for the efficient profiling of polar and charged metabolites in the metabolomics field. Compared to chromatographic-based separation techniques, CE-MS provides complementary metabolic information. Recent developments in interfacing designs resulted in an increased interest in the CE-MS technique for metabolomics, as these interfaces provide a significantly improved metabolic coverage in comparison to the conventional interface. There are many more developments in interfacing designs, which have not been covered in this chapter as their utility for metabolomics still needs to be assessed. Concerning the use of novel interfacing designs, the long-term performance of these approaches for metabolomics studies needs to be demonstrated. One way to achieve this is to perform an inter-laboratory metabo-ring study using the (various) CE-MS approaches, and this is indeed a step that we have recently initiated. Next to such a study, standardized CE-MS-based analytical workflows need to be developed including data analysis procedures. The first steps into this direction have already been taken, for example, with the design of the open-access software ROMANCE for converting migration times into electrophoretic mobilities. Support from vendors would also be very helpful in this context.

We foresee the metabolomics study of the highly polar fraction of the metabolome and of volume- and biomass-limited samples as the main application areas for CE-MS, especially the approaches utilizing a new interfacing design. In this context, an important sweet spot for CE-MS will be metabolic profiling of primary cells, cell culture extracts from 3D microfluidic systems, spheroids and liquid biopsies. Chiral metabolic profiling of biological samples will be another key application field.

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