Chapter 5
Strategies Involving Mass Spectrometry Combined with Capillary Electrophoresis in Metabolomics

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Abstract This chapter focuses on the important contribution of CE-MS in metabolomics, describing the nature of CE-MS coupling and the technical improvements that have led to the interfaces used in modern instrumentation. Moreover, it will discourse how the variety of electrolyte compositions and additives, which has conferred CE the exceptional selectivity of its multiple separation modes, has been handled to allow interfacing with MS without compromising ionization efficiency and the spectrometer integrity. Finally, the methodologies of CE-MS in current use for metabolomics will be discussed in detail. To verify the scope of CE-MS in clinical metabolomics, a myriad of representative applications has been compiled.

Keywords Metabolomics • Targeted metabolomics • Untargeted metabolomics • Clinical metabolomics • CE-MS • Capillary electrophoresis • CE-MS interfaces

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Abbreviations

ACE	Affinity capillary electrophoresis
ACS	Acute coronary syndrome
AD	Alzheimer's disease
ANN	Artificial neural network
ANOVA	Analysis of variance
APCI	Atmospheric pressure chemical ionization
APFO	Ammonium perfluorooctanoate
BGE	Background electrolyte
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
p-CEC	Pressure-assisted CEC
CESI	Capillary electrophoresis integrated to electrospray ionization
CGE	Capillary gel electrophoresis
CIEF	Capillary isoelectric focusing
CITP	Capillary isotachophoresis
CKD	Chronic kidney disease
CMC	Critical micelle concentration
CoA	Co-enzyme A
CRPS	Complex regional pain syndrome
CSF	Cerebrospinal fluid
CTAB	Cetyltrimethylammonium bromide
CVA	Canonical variate analysis
CZE	Capillary zone electrophoresis
DA	Discriminant analysis
DS	Dextran sulfate
EOF	Electroosmotic flow
ESI	Electrospray ionization
FAB	Fast atom bombardment
FDR	False discovery rate
FTICR	Fourier-transform ion cyclotron resonance
GC	Gas chromatography
HCA	Hierarchical cluster analysis
HF	Hydrogen fluoride
HILIC	Hydrophilic interaction liquid chromatography
^{1}H NMR	Proton nuclear magnetic resonance
HPLC	High-performance liquid chromatography
I.D.	Inner diameter
IS	Internal standard
IT	Ion trap
kNN	k-Nearest neighbors
LC	Liquid chromatography

LDA	Linear discriminant analysis
LLE	Liquid-liquid extraction
LOO-CV	Leave-one-out cross validation
M	Molecule
MALDI	Matrix-assisted laser desorption ionization
MCR-ALS	Multivariate curve resolution alternating least squares
MEKC	Micellar electrokinetic chromatography
MLR	Multiple linear regression
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSc	Multiple sclerosis
MSI	Multi-segment injection
O.D.	Outer diameter
OPLS	Orthogonal projections to latent structures
PB	Polybrene
PCA	Principal component analysis
PF	Partial filling
PKD	Polycystic kidney disease
PLS	Partial least square
PVA	Polyvinyl alcohol
PVS	Poly(vinyl sulfonate)
Q	Quadrupole
QC	Quality control
QqQ	Triple quadrupole
qRT-PCR	Quantitative real-time polymerase chain reaction
RPLC	Reversed-phase liquid chromatography
SAM	Significance analysis of microarrays
SDS	Sodium dodecyl sulfate
SHL	Sheath liquid
SPE	Solid-phase extraction
SRM	Selected reaction monitoring
SVM	Support vector machine
TEA	Triethylamine
TEDETAMA-co-HPMA	Copolymers of N-(2-hydroxypropyl) methacrylamide (HPMA) and the dendronic methacrylic monomer 2-(3-(Bis(2-(diethylamino)ethyl)amino)propanamido) ethyl methacrylate (TEDETAMA, derived from N,N,N′,N′-tetraethyldiethylenetriamine, TEDETA)
TOF	Time of flight
UPLC	Ultra-performance liquid chromatography
UTI	Urinary tract infection
UV	Ultraviolet radiation
VIP	Variable importance in the projection
VUR	Vesicoureteral reflux

5.1 The Niche of CE-MS in Metabolomics

Metabolomics, the analysis of the entire set of metabolites (metabolome), or a partial set of selected metabolites and/or substrates, expressed by an organism in preestablished conditions, via comparative experiments, has been the subject of irrefutable attention by the scientific community, since its inception in the late 1990s by Nicholson et al. and Fiehn [1, 2]. Both formats, untargeted (hypothesis generating) and targeted (hypothesis driven) metabolomics, are possible and have helped characterizing systemic responses of organisms to disease, pharmaceutical intervention, and dietary modulation [3–6]. Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy [7, 8] and mass spectrometry (MS) hyphenated with high- or ultra-performance liquid chromatography (HPLC or UPLC) and gas chromatography (GC) are the analytical platforms with prevalent use in the characterization of the metabolome [9–14]. A plethora of applications [15] with natural product-related [16, 17], nutritional [18–20], pharmaceutical [21], and clinical [22–26] importance have been compiled periodically. The choice of analytical technology applied in such studies is typically dependent upon the assessed class of chemical compounds, the cost of analysis, ease of sample preparation, and the requirement for sensitivity, specificity, and robustness. No single method enables complete coverage of the holistic metabolic information, and increasingly, metabolomics studies are adopting more than one analytical platform to augment the number of identified metabolites.

The particularities of metabolomics within the context of systems biology, as well as a general workflow of metabolomics studies from experimental design to biological validation, have been discussed thoroughly in Chap. 1. It is important to detail here the extent by which different analytical platforms approach the metabolome contents and how capillary electrophoresis is inserted in this context.

NMR has been the precursor technique for metabolomics and made a relevant contribution in the variety of application areas cited and referenced so far. This is mostly due to suitable performance characteristics, such as robustness, ease of data acquisition, and fairly wide metabolic coverage [1, 7, 8]. However, sensitivity and spectrum complexity have been issues in NMR metabolomics studies.

Nowadays, the high selectivity and sensitivity offered by MS platforms allowed MS to have conquered a sizeable niche in metabolomics, especially when the mass analyzer is hyphenated up front to a separation instrument [14]. Temporal separation of metabolites prior to detection is a desirable feature when complex matrices such as biological fluids and tissues are assessed.

GC-MS has been comprehensively explored for metabolomics since the very beginning [2, 14], with early studies in the context of plant metabolomics [16]. Although GC-MS is suited to assess the volatile portion of the metabolome, sample derivatization schemes [27] aiming primarily at volatility enhancement allowed GC

to reach a rather polar fraction in water-rich biofluids; for instance, carboxylic acids, amino acids, and biogenic amines can all be analyzed simultaneously in a single chromatographic run and ionization mode. Moreover, the high specificity associated with the resulting adducts allows the use of low-resolution mass spectrometers and the building of dedicated spectra libraries for compound identification [28]. Nevertheless, necessary sample derivatization schemes are time-consuming tasks and have limited the application of GC-MS to clinical protocols where only a small set of samples is under consideration.

Liquid chromatography-mass spectrometry (LC-MS) has been the premier technique in metabolomics for many years [9–14], despite the fact that to achieve the same metabolic coverage NMR does, multiple column chemistries must be screened. There are plenty of systematic studies where the information acquired from the more traditional reversed-phase (RPLC-MS) to the recently revisited hydrophilic interaction (HILIC-MS) modes is combined to promote a more thorough metabolic coverage (from nonpolar and/or moderately polar metabolites up to the ionic/polar ones) [29, 30]. The completion of human serum and urine metabolomes is a good example of the complementary information NMR and hyphenated MS analytical platforms offer [31, 32].

Considering the orthogonal separation mechanism provided by capillary electrophoresis (CE), it has emerged as a promising complementary technique to both liquid and gas chromatography for metabolic profiling of biological fluids as an impressive series of periodic review articles attest [33–56]. Intrinsic characteristics, such as high efficiency and resolution power, rapid analyses, and, most importantly, the ability to assess, without derivatization, the most polar and/or ionic compounds in the metabolome, have placed CE in an advantageous position. This chapter will therefore give a comprehensive overview of the state of the art in CE-MS technology, describing the methodologies in use for metabolomics and compiling representative applications of CE-MS in clinical metabolomics.

5.2 CE-MS

5.2.1 Onset and Pioneer Work

Although MS has currently achieved remarkable capacity to screen the composition of complex samples, in order to obtain relevant information about any biological system in a comprehensive manner as metabolomics studies do, it is recommendable to couple MS with different separation techniques and benefit from the three-dimensional information the hyphenated system imparts (retention and/or migration time, peak intensity, and mass-to-charge ratio).

Within the context of coupling separation techniques to MS, CE-MS was the last to be established, and interfacing the two platforms followed a timeline. While GC and HPLC were firstly registered around the 1950s and 1960s [57, 58], the first reports on electrophoresis effectively performed on capillary tube dimensions were registered in 1981, by Jorgenson and Lukacs [59, 60]. Before that, some authors had published electrophoretic separation on "quasi-capillary" dimensions, namely, Hjérten (using 300 μm i.d. capillary for the separation of inorganic ions, nucleotides, and proteins), Virtanen (using 200–500 μm i.d. capillaries), and Everaerts and collaborators, who first reported a completely automatized CE system using 100 μm i.d. capillaries [61–63].

Capillary zone electrophoresis (CZE) is the simplest and most commonly used CE mode due to the straightforwardness of background electrolyte (BGE) composition, principle of separation, and broad application to the analysis of diverse samples, containing from small ions to large biomolecules [64]. In CZE, analytes are separated according to differences in electrophoretic mobilities, which are dependent on the molecule/species charge-to-radius ratio and the medium viscosity. Neutral analytes are thus not separated by this mode, constituting one of the CZE main drawbacks. To overcome such limitations and to expand CE applicability, other CE modes have been developed, such as micellar electrokinetic chromatography (MEKC, where micelles are used as carriers to assess primarily the separation of neutral compounds) [65], capillary isoelectric focusing (CIEF, where separation of amphiprotic substances is conducted in a pH gradient) [66–68], capillary isotachophoresis (CITP, where discontinuous leading and terminating electrolytes are used to separate small molecules and ions) [69], capillary gel electrophoresis (CGE, which uses gels or entangled polymers to assess large molecules and polymers) [70], capillary electrochromatography (CEC, where packed capillary columns are used to explore additional solute-stationary phase interactions) [71], and affinity capillary electrophoresis (ACE, which explores biospecific interactions) [72], among other modes. In fact, the versatility of performing almost all different modes (CEC and certain formats of CGE are a few exceptions) in the same capillary format and in the same equipment, only requiring alteration of the BGE composition, constitutes one of the major advantages of CE as a separation technique. Additional characteristics of CE include high resolution and efficiency (a million plates can be achieved), low consumption of BGE (few μL per run), small sample volume (few nL per run), and relatively fast separations (less than 5 min in favorable cases).

The most frequently used detection scheme available in almost all commercial CE equipments is based on absorption of UV-visible radiation (CE-UV). The UV detector is usually built on-capillary. By removing a narrow portion of the polyimide that coats externally the capillary, a detection window of tenths of millimeters is created. Any compound containing a chromophore group that passes the detection window will absorb the UV-visible radiation focused on the capillary and give a signal. However, the optical length available for absorption is the capillary inner diameter (usually 50 or 75 μm). Comparing such dimensions with those presented by HPLC detection cells (in the order of cm), allied to the reduced sample volume

introduced into the CE capillary (in the order of nL), puts in evidence the reduced concentration sensitivity posed by CE-UV systems.

The analysis of biological samples generally requires the use of sensitive, selective, and universal detectors. CE-MS coupling has arisen as a valuable alternative to overcome sensitivity and also selectivity issues associated with the UV detector, since the MS detector provides online spectral information; in addition, it is universal, and rather sensitive, depending on the interface used in the coupling. However, two main issues must be addressed when coupling CE to MS, namely, the separation mode and the interface design.

The CE-MS coupling was registered for the first time in 1987 by Smith and collaborators [73], constituting the first description of what is now known as sheathless interface. In their pioneer work, a capillary electrophoresis system (operated under CZE mode) was coupled to a quadrupole mass spectrometer using electrospray ionization (ESI) for the analysis of quaternary ammonium salts. In their instrumental arrangement, the cathode (or low voltage end) of the CE capillary was inserted into a stainless steel capillary in order to establish both the CE and the ESI electrical circuits. The capillary inlet was immersed in a BGE reservoir, and an electroosmotically induced flow allowed the CE effluent to be directly introduced into the MS. Nitrogen gas was used as drying gas in countercurrent to the CE effluent to assist droplet desolvation. Higher separation efficiency was obtained by decreasing the sample plug and concentration. However, several restrictions were imposed by this first CE-MS arrangement: low flow requirements for spray stability, limited BGE composition, and issues related to the capillary preparation process, such as the need of several steps for metal deposition and erosion of the deposited metal, requiring replacement after a few days of operation. By addressing these problems, in the following year, Smith and col. published a new manuscript, where some improvements were reported [74]. The CE capillary cathode end was again inserted into a stainless steel tube, and few millimeters of the capillary were protruded outside. In addition, silver vapor was used to produce the metal deposit at the capillary end, providing a system with better mechanical strength and extended lifetime. As a result, improved efficiencies of ESI sampling and ion transmission were achieved. A broader range of compounds, such as amino acids, polypeptides, quaternary ammonium salts, and water-soluble vitamins, was analyzed, presenting better separation efficiency than reported previously. In the same year, Smith and col. described a completely new and improved ESI interface, where the contact at the CE terminus was replaced by a thin sheath of flowing liquid [75]. It was the birth of the sheath liquid interface. With this new arrangement, a qualitative improvement in ESI stability was reached, and more importantly, no special treatment was required to establish the electrical contact at the capillary end, allowing easy replacement of the capillary. This design also constituted the basis for implementation of other CE modes. It is important to recognize the merit and contribution of Smith's research group, who proposed in 1 year apart both CE-MS interface designs, which evolved into today's modern instrumentation [76].

Figure 5.1 depicts a schematic representation of the variety of CE mechanisms, ionization modes, and types of mass analyzers reported so far in CE-MS coupling.

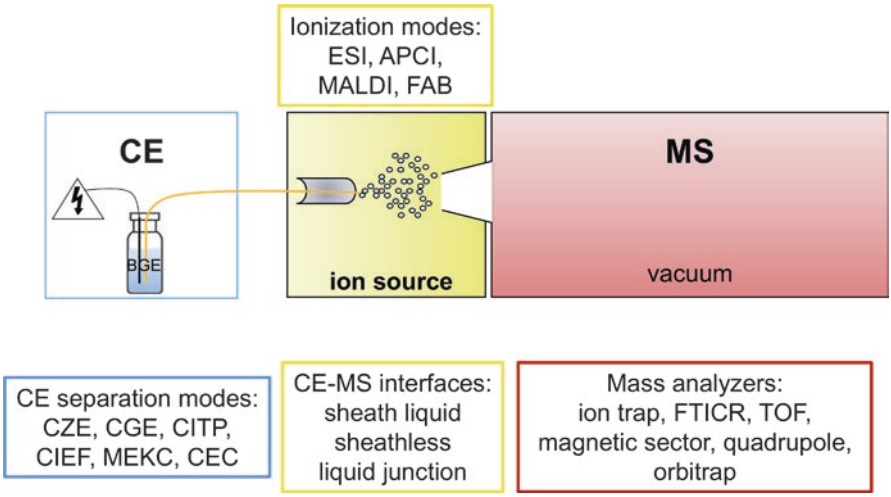

Fig. 5.1 Schematic diagram of CE-ESI-MS systems

CZE is undoubtedly the most commonly used separation mode in the CE-MS coupling, due to the easy manipulation of BGE composition (vide Table 5.1 for examples). In general, a simple buffer solution composed of volatile or semi-volatile acids or bases and corresponding salts is employed, e.g., formic or acetic acids, ammonia, and/or ammonium formate or acetate. However, the use of such simple BGE compositions restricts the pH range in which CE-MS separations can be performed. BGE additives may be required to improve the separation quality when resolution is compromised. The use of organic modifiers and cyclodextrins has been invoked, although the latter is known to hinder ionization efficiency and to contaminate the ion source and/or the mass analyzer ion optics, restricting its applicability.

MEKC has also been coupled to MS and provide the concurrent separation of neutral and ionic compounds, which interact with micelle compartments and/or surface to different extents. The solute McGown volume and the solute's ability to interact with electrolyte components via hydrogen bonding seem to be the determinant factors that explain retention. The surfactants commonly used in MEKC (SDS, CTAB, bile salts, etc.), which are added to the BGE in a concentration above their critical micelle concentrations (CMC), are generally nonvolatile species. Thus, depending on the concentration used, they might cause ionic suppression at the interface and contamination of the ion optics or capillary clogging if introduced into the MS system [147]. To circumvent these problems, the addition of volatile surfactants to the BGE has been recommended. Moreno-González et al. have employed ammonium perfluorooctanoate (APFO) as a semi-volatile surfactant for the separation of amino acids in human urine by MEKC-MS [148]. Separation of 20 amino acids (including leucine and isoleucine) with detectability in the ng mL^{-1} range was achieved with a BGE composed of 150 mmol L^{-1} APFO aqueous solution, adjusted to pH 9.0 with 14.2 mol L^{-1} ammonium hydroxide. At these pH

Table 5.1 Representative applications of CE-MS in clinical metabolomics

Ref.	Application	Matrix	Metabolomics	Metabolites	BGE	SHL	MS	Sample preparation	Data analysis	Other method
[77]	Acute coronary syndrome	Serum	Untargeted	Acetylcarnitines, amino acids	0.8 mol L^{-1} HFor in 10% MeOH	4 μL HFor in 50% MeOH	ESI(+)-TOF	LLE, ultracentrifugation	*t*-test, PCA, PLS-DA, OPLS-DA	LC-MS/MS
[78]	Aging	TTD mice urine	Untargeted	Amino acids and derivatives and acetylspermidine	2 mol L^{-1} HFor in 20% MeOH	50% MeOH with 0.1% HFor	ESI(+)-TOF	Mixed with MeOH, H_2O, and BGE; centrifuged	XCMS, PCA, PLS-DA	–
[79]	Alzheimer's disease *Progression*	CSF	Untargeted	Choline, arginine, histidine, dimethy-L-arginine, carnitine, creatine, valine, serine, and proline	0.5 mol L^{-1} HFor at pH 1.8	IPOH/H_2O (50%)	ESI(+)-TOF	Internal standards added to the CSF followed by ultrafiltration	Data analysis (Bruker Daltonics), Mzmine, PCA, LOO-CV, LDA, CVA	–
[80]	Alzheimer's disease *Progression*	Serum	Untargeted	Polar metabolites	0.8 mol L^{-1} HFor in 10% MeOH	1 mmol L^{-1} HFor in 50% MeOH	ESI(+)-TOF	LLE, ultracentrifugation	PLS-DA, VIP	–
[81]	Cancer *Bladder*	Urine	Untargeted	Amino acids and derivatives	0.8 mol L^{-1} HFor in 10% MeOH	1 mmol L^{-1} HFor in 50% MeOH	ESI(+)-TOF	Dilutionin H_2O (1:5)	ANOVA, OPLS-DA	LC-QTOF -MS
[82]	Cancer *Breast, mouth, and pancreas*	Saliva	Untargeted	Amino acids and derivatives, carboxylic acids, carnitine, amines, bile acid	1 mol L^{-1} HFor	50% MeOH with 0.5 μmol L^{-1} reserpine	ESI(+)-TOF	Dilution in H_2O (9:1) with IS	MassHunter (Agilent Technologies), XCMS, Douglas-Peucker algorithm, Steel-Dwass test, ANN. PCA, SVM, MLR	–
[83]	Cancer *Colon*	Cell	Untargeted	Amino acids and derivatives, amines, nucleoside, sugar	1 mol L^{-1} HFor	IPOH/H_2O (50%)	ESI(+)-TOF	LLE, ultrafiltration and lyophilization	XCMS, Welch *t*-test, Student's *t*-test	LC-MS (HILIC and RP)

(continued)

Table 5.1 (continued)

Ref.	Application	Matrix	Metabolomics	Metabolites	BGE	SHL	MS	Sample preparation	Data analysis	Other method
[84]	Cancer *Colon and stomach*	Tumor tissue	Targeted	Metabolites involved in glycolysis, pentose phosphate pathway, TCA, urea cycles, and amino acid and nucleotide metabolisms	1 mol L^{-1} HFor; 50 mmol L^{-1} AmAc, pH 8.5 (SMILE (+)-coated capillary) and 50 mmol L^{-1} AmAc, pH 7.5	50% MeOH with 0.5 μmol L^{-1} reserpine; 50% MeOH with 1 μmol L^{-1} reserpine and 5 mmol L^{-1} AmAc in 50% MeOH	ESI(+/−)-TOF	LLE, ultrafiltration and lyophilization	Wilcoxon test, multiExperiment Viewer	–
[85]	Cancer *Lung and prostate*	Tumor and surrounding tissues	Untargeted	Several	Cations: 1 mol L^{-1} HFor Anions: 50 mmol L^{-1} AmAc, pH 8.5	Commercial SHL	ESI(+/−)-TOF	LLE, ultracentrifugation	PCA	nanoLC-MS/MS
[86]	Cancer *Stomach*	Urine	Untargeted	Amino acids	1 mol L^{-1} HFor	0.1% HFor in 50% MeOH	ESI(+)-IT	Centrifugation, filtration	Mann-Whitney, ANOVA Kruskal-Wallis test, PCA	–
[87]	Cancer *Prostate*	Urine	Targeted	Sarcosine, L-proline, l-cysteine, L-leucine, L-glutamic acid, and L-kynurenine	0.4% HFor in 50% MeOH and 0.2% HFor in 50% MeOH	–	ESI(+)-IT	SPE (Strata-X strong cation cartridge) and lyophilization	–	–
[88]	Chronic kidney disease	Plasma	Untargeted		1 mol L^{-1} HFor; 50 mmol L^{-1} AmAc, pH 8.5 (SMILE (+)-coated capillary)	5 mmol L^{-1} AmAc in 50% MeOH	ESI(+/−)-TOF	LLE, ultrafiltration and lyophilization	Spearman's rank correlation, Likehood function	–

[89]	Complex regional pain syndrome	Urine	Untargeted	Amino acids and derivatives, carboxylic acids, amines	1 mol L^{-1} HFor pH 1.8 (PB-PVS coating)	0.1 % HFor in 50% MeOH	ESI(+)-TOF	Dilution 1:1 with BGE	XCMS, PCA, PLS-DA	MEKC
[90]	Data processing *Clustering algorithm*	Urine	Untargeted	Amino acids, organic acids, nucleotides	1 mol L^{-1} HFor adjusted with NH_4OH to pH 2.4 (TEDETAMA-co-HPMA copolymer-coated capillary)	50% IPOH	ESI(−)-TOF	Filtration	Hierarchical agglomerative cluster analysis (new algorithms)	–
[91]	Data processing *Missing values*	Plasma	Untargeted	Several	0.8 mol L^{-1} HFor in 10 % MeOH	1 mmol L^{-1} HFor in 50% MeOH	ESI(+)-TOF	LLE, ultracentrifugation	Zero, median, ½ minimum and kNN imputation methods, *t*-test, and Mann-Whitney test	–
[92]	Diabetes mellitus *Type 1*	Urine	Untargeted	Protein and amino acid metabolism	0.8 mol L^{-1} HFor in 10 % MeOH	1 mmol L^{-1} HFor in 50% MeOH	ESI(+)-TOF	Dilution with H_2O (10x) and centrifugation	PCA, OPLS-DA, *t*-test	LC-MS (plasma)
[93]	Diabetes mellitus *Type 2*	Serum	Untargeted	Amino acids, citrulline, acetylcarnitine	0.8 mol L^{-1} HFor in 10 % MeOH	4 µL HFor in 50% MeOH	ESI(+)-TOF	LLE, ultracentrifugation	*t*-test, Mann-Whitney test, Mann-Kendall trend analysis	–
[94]	Dilated cardiomyopathy	Heart tissue	Untargeted	Charged metabolites	Cations: 1 mol L^{-1} HFor Anions: 50 mmol L^{-1} AmAc, pH 8.5	5 mmol L^{-1} AmAc in 50% MeOH containing 0.1 µmol L^{-1} hexakis	ESI(+/−)-TOF	Homogenization with MeOH	PCA, *t*-test	LC-MS/MS LC-TOF-MS

(continued)

Table 5.1 (continued)

Ref.	Application	Matrix	Metabolomics	Metabolites	BGE	SHL	MS	Sample preparation	Data analysis	Other method
[95]	Exercise training	Plasma	Untargeted	L-carnitine,glutathionyl-L-cysteine, hypoxanthine,O-acetyl-L-carnitine	1 mol L^{-1} HFor in 15 % ACN	0.1 % HFor in 60% MeOH	ESI(+)-TOF	Dilution with 200 mmol L^{-1} AmAc, ultrafiltration	*t*-test, PCA, HCA, PLS-DA, two-way ANOVA	–
[96]	Fatty liver disease *Nonalcoholic related*	Serum	Untargeted	Sulfated steroids	50 mmol L^{-1} AmAc pH 8.5 (COSMO (+)-coated capillary)	5 mmol L^{-1} AmAc in 50% MeOH containing 0.1 µmol L^{-1} hexakis	ESI(−)-TOF	LLE, ultracentrifugation	Steel-Dwass test	LC-TOF-MS
[97]	Gastric injury *Aspirin induced*	Serum, and stomach tissue	Untargeted	TCA cycle, β-oxidation, collagen metabolism	Cations: 1 mol L^{-1} HFor Anions: 50 mmol L^{-1} AmAc, pH 8.5 (COSMO (+)-coated capillary)	5 mmol L^{-1} AmAc in 50% MeOH containing 0.1 µmol L^{-1} hexakis	ESI(+/−)-TOF	LLE, ultrafiltration	PCA, PLS-DA, ANOVA, Dunnett's test	–
[98]	Hepatitis	Tissue	Untargeted	Compounds related to glutathione biosynthesis	1 mol L^{-1} HFor; 50 mmol L^{-1} AmAc, pH 8.5 (SMILE (+)-coated capillary)	5 mmol L^{-1} AmAc in 50% MeOH containing 20 µmol L^{-1} PEPIS and 1 µmol L^{-1} reserpine	ESI(+/−)-TOF	Centrifugation, filtration and lyophilization	Douglas-Peucker algorithm, Reijenga function	–
[99]	Hepato-cellular carcinoma	Serum	Untargeted	Amino acids, organic acids, amines, sugar phosphates	Cations: 1 mol L^{-1} HFor Anions: 50 mmol L^{-1} AmAc pH 8.5	5 mmol L^{-1} AmAc in 50% MeOH containing 0.1 µmol L^{-1} hexakis	ESI(+/−)-TOF	LLE, ultracentrifugation	PLS-DA, HCA, Wilcoxon Mann-Whitney test, FDR	–

[100]	Huntington's disease *Progression*	Plasma	Untargeted	Protein metabolism, prostaglandins, thromboxanes, lipoxins, and leukotrienes	50 mmol L^{-1} HAc and 50 mmol L^{-1} HFor adjusted to pH 3.5 with ammonia	0.05% HFor in 60% IPOH	ESI(+)-TOF	Protein precipitation, ultracentrifugation, SPE online	MCR-ALS, PLS-DA	–
[101]	Hypercholesterolaemia *Diet induced*	Plasma	Untargeted	Amino acids and derivatives, fatty acids esters	0.8 mol L^{-1} HFor in 10% MeOH	1 mmol L^{-1} HFor in 50% MeOH	ESI(+)-TOF	LLE, ultracentrifugation	PCA, PLS-DA	LC-MS, GC-MS
[102]	Inborn errors of metabolism	Dried blood spot	Targeted	Amino acid and acylcarnitine	1.4 mol L^{-1} HFor, pH 1.8	1:1 MeOH/H_2O with 0.1% HFor	ESI(+)-IT	LLE, ultrafiltration and dilution with AmAc solution	–	–
[103]	Liver disease	Serum	Untargeted	γ-glutamyl, dipeptides, transaminases, and methionine sulfoxide	50 mmol L^{-1} AmAc, pH 8.5 (COSMO (+)-coated capillary)	5 mmol L^{-1} AmAc in 50% MeOH and 0.1 µmol L^{-1} hexakis	ESI(−)-TOF	–	Kruskal-Wallis test and Dunn's post-test, Mann-Whitney test, MLR	LC-TOF-MS and LC-MS/MS
[104]	Liver disease	Serum, and liver tissue	Targeted	γ-glutamyl peptides	200 mmol L^{-1} HAc pH 3.3	0.5 mmol L^{-1} AmAc in 50% MeOH	ESI(+)-QqQ	LLE, ultracentrifiugation	–	LC-MS/MS
[105]	Liver injury *Alcohol related*	Plasma	Untargeted	Amino acids, guanidinosuccinate	Cations: 1 mol L^{-1} HFor Anions: 50 mmol L^{-1} AmAc, pH 8.5 (COSMO (+)-coated capillary)	Cations: 0.5 µmol L^{-1} reserpine in 50% MeOH Anions: 5 mmol L^{-1} AmAc in 50% MeOH containing 0.1 µmol L^{-1} hexakis	ESI(+/−)-TOF	LLE, ultracentrifugation	Linear regression analysis, Benjamini and Hochberg's FDR	–

(continued)

Table 5.1 (continued)

Ref.	Application	Matrix	Metabolomics	Metabolites	BGE	SHL	MS	Sample preparation	Data analysis	Other method
[106]	Lung injury *Ventilator induced*	Plasma	Untargeted	Organic amines, amino acids, and their derivatives, carnitines	0.8 mol L^{-1} HFor in 10% MeOH	4 μL HFor in 50% MeOH	ESI(+)-TOF	SPE (phospholipids and proteins removal) Ultracentrifugation	*t*-test, PLS-DA	
[107]	Metabolic disorders	Urine and blood spots	Targeted	Carnitines, carboxylic acid, creatinine, and galactose	20 mmol L^{-1} AmAc, pH 8.5	2 mmol L^{-1} AmAc in 50% MeOH	ESI(−)-QqQ	Blood spots: solid-liquid extraction; urine: filtration (0.45 μm)	–	–
[108]	Metabolite profiling *Estrogen speciation*	Urine	Targeted	Estrogens	50 mmol L^{-1} ammonium bicarbonate, pH 9.5	5 mmol L^{-1} ammonium bicarbonate in 80% MeOH	ESI(−)-TOF	Dilution (10x)	–	–
[109]	Metabolite profiling *Isobaric labeling*	Urine	Targeted	Amine-containing metabolites	0.2% HFor in 50% MeOH	0.2% HFor in 50% MeOH	ESI(+)-QTOF	Ultrafiltration, labeling with 4-plex DiLeu	–	nanoLC-ESI-MS/MS
[110]	Metabolite profiling *Kynurenic pathway*	CSF	Targeted	Tryptophan metabolites	5 mmol L^{-1} AmAc in 5% ACN pH 9.7	50% MeOH	ESI(+)-TOF	Diluted (5×), add 50% ACN	–	–
[111]	Metabolite profiling *Thiol speciation*	Plasma	Targeted	Thiols	1 mol L^{-1} HFor, pH 1.8	MeOH/H_2O (60:40%) with 0.1% HFor	ESI(+)-IT	Diluted 3-fold with 200 mmol L^{-1} AmAc, pH 5 and 20 mol L^{-1} Ala-Ala	RRF, MLR, k-fold cross validation	–
[112]	Method optimization *Additives for BGE and SHL*	Urine	Targeted	Amino acids and derivatives, carboxylic acids, nucleoside, sugar phosphate, purine	25 mmol L^{-1} TEA (pH 11.7) (PB-DS-PB-coated capillary)	50% MeOH with 5 mmol L^{-1} TEA	ESI(−)-TOF	Centrifuged and mixed with BGE (1:1)	DataAnalysis (Bruker Daltonics)	–
[113]	Method optimization *Capillary coating*	CSF, plasma and urine	Untargeted	Organic acids, amino acids	1 mol L^{-1} HFor pH 1.8 (PB-PVS-coated capillary)	50% MeOH with 0.1% HFor	ESI(+)-TOF	CSF and plasma: no preparation; urine: mixed with BGE and centrifuged	–	CE-UV

[114]	Method optimization *Capillary coating*	Urine	Untargeted/ targeted	Nucleosides, amino acids, carboxylic acids, nucleotides, amines	1 mol L^{-1} HFor; Anion: 25 mmol L^{-1} AmAc, pH 9 (PB-PVS and PB-DS-PB coated capillaries for both modes)	50 % MeOH with 0.1 % HForand 50 % MeOH with 0.1 % concentrated NH_4OH	ESI(+/−)-TOF	Mixed with BGE (1:1) for cation analysis or with H_2O (1:1) for anion	–	CE-UV
[115]	Method optimization *Capillary coating*	Urine	Untargeted/ targeted	Tyramine, dopamine, creatinine, hippuric acid, glutathione, proline betaine, and amino acids	1 mol L^{-1} HFor pH 2 (PB-DS-PB coating)	50 % MeOH with 0.1 % HFor	ESI(+)−TOF	Mixed with BGE (1:1) and centrifuged	XCMS, PLS-DA, PCA	UPLC-MS
[116]	Method optimization *Interface*	Urine	Untargeted	Amino acids and derivatives, amines, nucleic acids, and small peptides	1.7 molL^{-1} (10 %) HAc	Sheathless	ESI(+)-TOF	Mixed with IS and H_2O (1:1:8) and ultrafiltered	MassHunter (Agilent Technologies), XCMS, Douglas-Peucker algorithm, Steel-Dwass test	–
[117]	Method optimization *Interface*	Urine	Untargeted/ targeted	Amino acids and derivatives, carnitines, nucleosides, creatinine, vitamins	10 % HAc	Sheathless	ESI(+)-TOF	Mixed with BGE (1:1) and centrifuged	DataAnalysis (Bruker Daltonics)	–
[118]	Method optimization *Interface*	Mouse CSF, plasma and urine	Untargeted/ targeted	Purine, amino acids and derivatives, choline, creatinine	10 % HAc	Sheathless	ESI(+)-TOF	Diluted with H_2O (1:1)	–	–
[119]	Method optimization *Overall conditions*	Urine	Targeted	Dopamine, norepinephrine, epinephrine, 3-methoxytyramine, normetanephrine, metanephrine	1 % HAc, pH 2.8 (PVA-coated capillary)	MeOH:H_2O (75:25) with 0.1 % HAc	ESI(+)-TOF	SPE on Oasis MXC cation-exchange cartridge	–	CE-UV

(continued)

Table 5.1 (continued)

Ref.	Application	Matrix	Metabolomics	Metabolites	BGE	SHL	MS	Sample preparation	Data analysis	Other method
[120]	Method optimization *Overall conditions*	Urine	Untargeted/ targeted	Nucleosides, amino acids, carboxylic acids, sugars, nucleotides, monoamine	50 mmol L^{-1} HAc and 50 mmol L^{-1} HFor at pH 2.5	MeOH:H_2O (80:20) with 0.1 % HAc and IPOH/ H_2O (60:40 %) with 0.5 % ammonia	ESI(+/−)-IT	Centrifugation, filtration and lyophilization	–	CE-UV
[121]	Method optimization *Overall conditions*	Plasma	Targeted	Carnitines	200 mol L^{-1} ammonium formate, pH 2.5	MeOH:H_2O (70:30) with 0.1 % HFor	ESI(+)-IT	Deproteinized with cold ACN (1:5) and centrifuged	–	–
[122]	Method optimization *Pt needle sprayer*	Mouse liver tissue	Targeted	Metabolites in glycolysis, pentose phosphate, and TCA pathways	50 mmol L^{-1} AmAc, pH 8.5 (COSMO (+) coated capillary)	5 mmol L^{-1} AmAc in 50% MeOH with 0.1 µmol L^{-1} hexakis	ESI(−)-TOF	LLE, ultrafiltration and lyophilization	MZmine	–
[123]	Method optmization *Overall conditions*	Urine	Targeted	Amino acids	1 mol L^{-1} HFor	5 mmol L^{-1} AmAc in 50% MeOH	ESI(+)-QqQ	Dilution with H_2O (1:5)	–	–
[124]	Migraine *Cortical spreading depression*	Plasma	Untargeted	Lysine, pipecolic acid	10 % HAc (neutrally coated capillary)	Sheathless	ESI(+)-TOF	Protein precipitation with ethanol	PCA, PLS-DA, OPLS-DA	LC-MS/MS
[125]	Model organism *Bacteria*	*Bacillus subtilis*cells	Targeted	Intermediates of glycolysis and the TCA cycle	50 mmol L^{-1} AmAc, pH 9 (SMILE (+)-coated capillary)	5 mmol L^{-1} AmAc in 50% MeOH	ESI(−)-IT	LLE, ultrafiltration and lyophilization	–	–
[126]	Model organism *Bacteria*	*Bacillus subtilis*cells	Targeted	Citrate isomers, nucleotides, dinucleotides, and coenzyme A compounds	50 mmol L^{-1} AmAc, pH 7.5 (DB-1-coated capillary)	5 mmol L^{-1} AmAc in 50% MeOH	ESI(−)-MS pressure assisted	LLE, ultrafiltration and lyophilization	–	–

[127]	Model organism *Bacteria*	*Bacillus subtilis*cells	Untargeted/ targeted	Nucleosides, amino acids, organic acids, sugars, nucleotides, monoamine; compounds involved in glycolysis, TCA cycle, and pentose phosphate pathways	1 mol L^{-1} HFor; 50 mmol L^{-1} AmAc, pH 8.5 (SMILE (+)-coated capillary) and 50 mmol L^{-1} AmAc, pH 7.5	5 mmol L^{-1} AmAc in 50% MeOH	ESI(+/−)-IT	LLE, ultrafiltration and lyophilization	–	–
[128]	Model organism *Bacteria*	*Escherichia coli* cells	Untargeted	Nucleosides, amino acids, organic acids, sugars, nucleotides, monoamine	80% 20 mmol L^{-1} AmAc pH 9.5: 20% IPOH	Sheathless	ESI(−)-QIT	LLE, ultrafiltration and lyophilization	–	Direct infusion ESI-MS
[129]	Model organism *Bacteria*	*Escherichia coli* cells	Untargeted/ targeted	Nucleosides, amino acids, carboxylic acids, nucleotides, amines, sugars	50 mol L^{-1} AmAc, pH 8.7 in 5% MeOH	20 mmol L^{-1} NH_4OH in 50% IPOH	ESI(−)-TOF	LLE, ultrafiltration and lyophilization	QuantAnalysis (Bruker Daltonics) and MZmine	GC-MS
[130]	Model organism *Cell - single cell*	Thalamic tissue	Untargeted	GABA	1% HFor	0.1% HFor in 50% MeOH	ESI(+)-QTOF	LLE	–	–
[131]	Model organism *Cell*	Colon adenocarcinoma HT-29 cell line	Untargeted	Polyamines pathway	3 mol L^{-1} HFor	50% IPOH	ESI(+)-TOF	LLE, ultracentrifugation	PCA, ANOVA	–
[132]	Model organism *Cell*	U-87 MG glioblastoma cell line	Untargeted	Organic acids, sugar phosphates, and nucleotides	10% HAc pH 2.2	Sheathless	ESI(−)-TOF	Lysis, LLE	–	–
[133]	Model organism *Fish*	*Zebrafish* embryo	Untargeted	Cationic metabolites	10% HAc	0.1% HFor in 50% MeOH or 0.1% HAc in 75% IPOH (flow-through micro-vial interface)	ESI(+)-QTOF	Mechanical lysis, ultrafiltration	–	–

(continued)

Table 5.1 (continued)

Ref.	Application	Matrix	Metabolomics	Metabolites	BGE	SHL	MS	Sample preparation	Data analysis	Other method
[134]	Model organism *Parasite*	*Fasciola hepatica* tissue	Untargeted/ targeted	Organic amines, amino acids	0.8 mol L^{-1} HFor at pH 1.8 in 20% MeOH	0.5% HFor in 70% MeOH	ESI(+)-QTOF	LLE	XCMS, in-house script Matlab,	UPLC-MS (RP and HILIC)
[135]	Multiple sclerosis *Effect of methionine enkephalin*	Glioma cell line (C6, RG2, H4, U251,U87)	Untargeted	Amino acids, glycylglycine	Cations: 1 mol L^{-1} HFor Anions: 50 mmol L^{-1} AmAc, pH 8.5	50% MeOH containing 0.1 µmol L^{-1} hexakis	ESI(+/−)-TOF	LLE	HCA, SAM	qRT-PCR, flow cytometry analysis
[136]	Myopia	Vitreous humor	Untargeted	Methylation process metabolites, acetylcarnitines, amino acids	0.8 mol L^{-1} HFor in 10% MeOH	1 mmol L^{-1} HFor in 50% MeOH	ESI(+)-TOF	Dilution with H_2O (5x)	OPLS-DA	LC-MS
[137]	Neurodegenerative dementia	Serum, saliva	Untargeted	TCA cyclic metabolites, amino acids and derivatives, creatinine	1.7 mol L^{-1} HAc	Sheathless	ESI(+)-TOF	Ultracentrifugation	PCA, PLS-DA	–
[138]	Osteoarthritis	Urine	Targeted	Amino acids	2 mol L^{-1} HFor with 20% MeOH	50% IPOH with 0.1% HFor	ESI(+)-TOF	Spiked urine	PCA	–
[139]	Polycystic kidney disease	Plasma	Untargeted	Amino acids and derivatives, carboxylic acids, nucleoside, amines, carnitines	1 mol L^{-1} HFor, and 50 mmol L^{-1} AmAc, pH 8.5 (COSMO (+)-coated capillary)	50% MeOH with 0.1 µmol L^{-1} hexakis	ESI(+)-TOF	LLE, ultrafiltration and lyophilization	Unpaired *t*-test	–

[140]	Sample handling *Multi-segment injection*	Plasma	Untargeted	Amino acids and derivatives	1 mol L^{-1} HFor with 15% ACN	MeOH:H_2O (60:40) with 0.1% HFor	ESI(+)-TOF	Dilution with AmAc (4x) and ultracentrifugation	–	–
[141]	Sample handling *Processing and storage*	Serum, plasma	Untargeted	Charged metabolites	Cations: 1 mol L^{-1} HFor Anions: 50 mmol L^{-1} AmAc, pH 8.5 (COSMO (+) capillary)	Cations: 50% MeOH containing 0.1 µmol L^{-1} hexakis Anions: 5 mmol L^{-1} AmAc in 50% MeOH containing 0.1 µmol L^{-1} hexakis	ESI(+/−)-TOF	LLE, ultrafiltration	PCA, *t*-test	–
[142]	Schizophrenia	Plasma	Untargeted	Creatine, betaine, organic acids, homocysteine	Cations: 1 mol L^{-1} HFor Anions: 50 mmol L^{-1} AmAc, pH 8.5 (COSMO (+)-coated capillary)	Cations: 0.5 µmol L^{-1} reserpine in 50% MeOH Anions: 5 mmol L^{-1} AmAc in 50% MeOH containing 0.1 µmol L^{-1} hexakis	ESI(+/−)-TOF	LLE, ultracentrifugation	Mann-Whitney test, step-wise DA	–
[143]	Sepsis	Rat lung tissue	Untargeted	Amino acids, amines, carnitines	0.8 mol L^{-1} HFor in 10% MeOH	4 µL HFor in 50% MeOH	ESI(+)-TOF	LLE, ultracentrifugation	*t*-test, PLS-DA	LC-MS, GC-MS
[144]	Urinary tract infection	Urine	Untargeted/ targeted	Amino acids and derivatives	1 mol L^{-1} HFor pH 1.8 (PB-PVS-coated capillary)	0.1% HFor in 50% MeOH	ESI(+)-TOF	Mixed with BGE and centrifuged	ANOVA, PLS-DA, VIP	–

(continued)

Table 5.1 (continued)

Ref.	Application	Matrix	Metabolomics	Metabolites	BGE	SHL	MS	Sample preparation	Data analysis	Other method
[145]	Vesicoureteral reflux	Urine	Targeted	Amino acids	0.80 mol L^{-1} HFor pH 1.96 in 15 % MeOH	0.50 % HFor in 60 % MeOH	ESI(+) -IT	Dilution	–	–
[146]	Xenobiotic exposure	Urine	Untargeted	Paracetamol sulfate, glucoronide, cystein, and paracetamol glucoronide	20 mmol L^{-1} ionic strength HFor/ammonium formate buffer, pH 3 (PolyE-323-coated capillary) and 20 mmol L^{-1} ionic strength NH_4OH/AmAc buffer, pH 9	80 % IPOH and 20 % BGE	ESI(+/−)-QqQ	–	In-house developed Matlab procedures and PCA	–

All percentages and proportions are expressed in v/v
ACN acetonitrile, *AmAc* ammonium acetate, *HAc* acetic acid, *HFor* formic acid, *IPOH* isopropanol, *MeOH* methanol

and concentration conditions, a strong electroosmotic flow (EOF) is observed, the surfactant is totally deprotonated (perfluorooctanoic acid has a pK_a of 2.8), and APFO micelles are formed (CMC is 25 mmol L^{-1}). With the exception of lysine and arginine, at the pH range from 7 to 9, amino acids are either negatively charged or neutral, which enhances their interaction with micelles. The authors observed that analyte resolution under the optimized conditions is a result of micelle partitioning and electrophoresis. Therefore, the most negatively charged amino acids were attracted to the anode, presenting low mobilities, while the positively charged amino acids interact electrostatically with the micelle surface, also showing long migration times. Finally, this method presented an improved selectivity when compared to a standard CZE-MS method and required a simple dilution of the urine sample with BGE prior to introduction into the CE system.

An interesting strategy to couple MEKC to MS is to partially fill the CE capillary with a BGE containing surfactant, whereas the remaining portion of the capillary is filled with a regular BGE, compatible with the MS system. In the partial filling technique (PF-MEKC-MS), the analytes are then primarily separated by interactions with micelles in the surfactant BGE length and reach the CE-MS interface before the surfactant does, when the current is interrupted. Sirén et al. have developed both PF-MEKC-UV and PF-MEKC-MS methods for the separation of endogenous low-hydrophilic steroids in plasma and urine samples [149]. For the PF-MEKC-MS method, the micellar solution was composed of 29.3 mmol L^{-1} SDS and 1.1 mmol L^{-1} sodium taurocholate in 20 mmol L^{-1} ammonium acetate at pH 9.68. Analytes that partially co-migrated after passing the surfactant BGE length were resolved in the MS operated at selected reaction monitoring (SRM) mode, resulting in the separation of eight analytes within 9 min.

CEC-MS has also been successfully used for the analysis of clinical samples. CEC development aimed at joining the high efficiency of CE (since the mobile phase flows through the capillary by EOF action, instead of pump pressure) with the high selectivity and peak capacity offered by the stationary phase in liquid chromatography. Therefore, a stationary phase must be introduced into the CE capillary, which may be one of the main experimental challenges of the CEC technique. The literature reports some options to this task, such as slurry packing (which may form bubbles in the separation bed and demands insertion of frits into the capillary ends to retain the stationary phase), open-channel CEC (where the inner capillary wall is functionalized), and in situ polymerization of a monolithic phase, which is the preferred strategy. Blas and McCord have performed the analysis of urine samples by CEC-MS to quantify traces of ten benzodiazepines [150]. A monolith based on porous acrylate was used. An online pre-concentration step (stacking consisting of the injection of a large amount of sample – 15 min at 12 bar – dissolved in aqueous medium) and the use of a TOF mass analyzer were required to obtain high sensitivity and specificity. To ensure that only hydrophobic interactions between analytes and the monolith occur, analyses were performed at pH 7.0, using 5 mmol L^{-1} ammonium acetate as BGE. Under these conditions, analytes at 1 ng mL^{-1} in urine samples could be quantified. One of the main problems encountered in CEC is bubble formation due to Joule heating, which may cause column dry out and current

interruption. To circumvent these problems, pressure-assisted CEC (*p*-CEC), where an extra pressure flow matches the EOF, coupled with ESI-QTOF-MS via a sheathless interface has been proposed for metabolomics profiling of urine samples [151]. The optimized method was successfully applied in the contrast of lung cancer patients and healthy subjects. Among 16 discriminant metabolites, three glutamine conjugates, including phenylacetylglutamine, acylglutamine C8:1, and acylglutamine C6:1, were identified.

To our knowledge, other CE modes in CE-MS couplings, such as CIEF-MS and CGE-MS, have not been applied in clinical metabolomics studies, and they will not be covered in this chapter.

5.2.2 CE-MS Interfaces

An important aspect to be considered when coupling CE to MS is the interface itself. Although many ionization schemes have been tested to date (Fig. 5.1), electrospray ionization (ESI) has been the ionization mode of choice, since it transfers ionizable analytes from the liquid phase to the gas phase, and it allows the analyses of high molecular-mass molecules by inducing formation of multiple charges (reduced *m/z* values). The development of CE-ESI-MS interfaces has mirrored the established LC-ESI-MS couplings. However, the reduced CE flow and the CE electric circuit (which must be closed at the CE capillary outlet or at the MS entrance) had to be regarded.

There are three main configurations for coupling CE to MS: coaxial sheath liquid interface, liquid junction interface, and sheathless interface. The formers are also called microspray interfaces while the latter is referred as nanospray interface [55, 152–155]. Some of the modern interface couplings used in CE-MS technology are schematically represented in Fig. 5.2.

The coaxial sheath liquid interface has gained great acceptance in CE-MS applications because it promotes a good spray stability, resulting in great robustness (Fig. 5.2a, b). Basically, the CE capillary outlet is introduced into a concentric tube where a sheath liquid (SHL) is pumped at nano- to microliter min^{-1} range. A third concentric tube may be introduced in order to conduct a nebulizer gas, assisting in the spray formation. One main drawback of sheath liquid interfaces is that the electrophoretic effluent (typical flow rate between 10 and 300 μL min^{-1}) is mixed to the SHL (typically 1–10 μL min^{-1}) at the capillary end, leading to sample dilution and consequent reduced detection sensitivity (one to two orders of magnitude decrease, depending on the BGE pH). Optimization of BGE composition as well as SHL flow and composition must therefore be carefully investigated in order to improve the ESI process and overall system detectability [55]. Nevertheless, the selection of both BGE and SHL is restricted to volatile or semi-volatile compounds, and routinely used salts in CE separations, such as borate and phosphate, must be avoided. Mixtures of organic solvents –

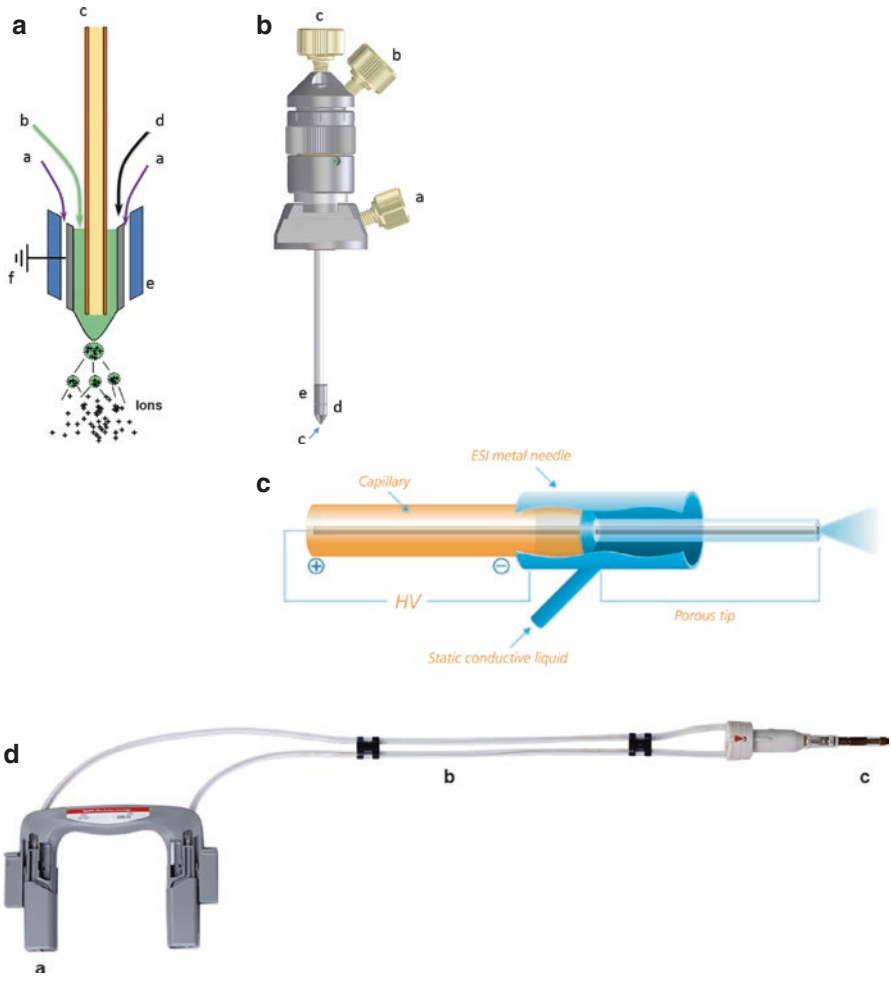

Fig. 5.2 Modern interfaces for CE-MS: coaxial sheath liquid (**a**, **b**) and sheathless (**c**, **d**) designs. Legends: (**a**) pictorial representation of Agilent coaxial sheath liquid CE-MS interface, (*a*) nebulizing gas, (*b*) sheath liquid, (*c*) CE capillary with BGE, (*d*) stainless steel spray needle with 0.4 mm i.d. and 0.5 mm o.d., (*e*) outer tube, and (*f*) ground connection; (**b**) engineering sketch of the coaxial sheath liquid CE-MS interface (graphics courtesy from Agilent Technologies) (reprinted with permission from Ref. [55]); (**c**) pictorial representation of Sciex sheathless CE-MS interface, (*a*) CE capillary inlet, (*b*) static conductive liquid capillary, and (*c*) sprayer porous tip; (**d**) engineering sketch of the CESI interface and cartridge (Photos are provided courtesy of AB Sciex Pte. Ltd. Operating as Sciex)

such as acetonitrile, methanol, and isopropanol – water, and weak acids or bases solutions (for positive and negative ESI, respectively) are generally considered for SHL [156]. Evaluation of the stability of CE-ESI-MS methods with sheath liquid interface may be derived from inter- and intraday precision measurements,

where the variation of peak areas of analytical standards spiked in body fluids is considered [157].

The sheath liquid interface position may be linear or orthogonal to the MS system. The main advantage offered by the latter geometry is that contamination or clogging of the MS inlet is less prone to occur and the choice of BGE composition is less critical, allowing the use of less volatile salts. Moreover, since the ESI voltage is applied at the MS entrance, charged species from the CE system are directed to the MS by electrostatic interaction, leading to higher detectability than in linear interfaces [158]. In fact, this is the most suitable and robust way to isolate the CE and the ESI electrical circuits.

The liquid junction interface is also based on a system supported by a sheath liquid [159]. However, mixing of BGE and SHL occurs far from the MS entrance, within a reservoir. Actually, the CE capillary ends inside this reservoir, and, in the opposite side, an electrospray needle is positioned within a distance ranging from 10 to 25 μm. With this geometry, the CE and the ESI electrical circuits operate individually, and the BGE selection may be performed independently of the MS restrictions. In addition, replacement of the ESI needle may be easily accomplished and does not affect the CE capillary. However, there are three main disadvantages that cause the scarce application of this interface: (i) the right alignment between the CE capillary and the ESI needle is laborious, (ii) the dead volume within the reservoir leads to band broadening (with consequent loss of separation efficiency), and finally, (iii) bubble formation on the CE capillary outlet often occurs, due to electrolysis reactions, resulting in current drop.

A recent variation of sheath liquid interfaces has been proposed by Chen and collaborators, named flow-through micro-vial interface [160]. The main characteristic of this interface is that the electrical circuits and flow rate requirements of the separation and ionization processes are decoupled. The authors have used a stainless steel hollow needle with optimized geometry to surround the CE capillary end. Therefore, the inner side of the needle works as the CE outlet vial, while the outer side is used as the ESI emitter. The CE capillary end is inserted into the needle until its outer diameter meets the dimension of the inner side of the needle. Both needle and CE capillary are connected to a tee union, where a second capillary is orthogonally attached in order to deliver the SHL into the needle. Typical flow rates of the SHL are as low as 0.1 mL min^{-1}, which reduces considerably the dilution of the CE effluent at the capillary end, when compared to regular sheath liquid interfaces, improving sensitivity. Another characteristic of the proposed interface is the possibility of using capillaries with any type of surface modification (such as neutral-coated capillaries for protein analysis, for instance) or even no pretreatment. The performance of the flow-through micro-vial interface comparatively to the conventional sheath liquid interface was evaluated by Lindenburg et al. in the profiling of cationic metabolite standards, exhibiting a fivefold improvement in terms of detection limits [133].

Sheathless interfaces transfer directly the CE effluent into the MS system, avoiding sample dilution and, consequently, present the best detectability among the CE-MS interfaces [161]. The main requirement of such interface is to close the CE

electrical circuit at the capillary end and simultaneously to afford electrical potential to the ESI. Considering that coaxial sheath flow interfaces have been developed after LC-MS interfaces, the possibility to develop an interface exclusively used in CE-MS coupling has arisen the interest of many research groups. For this reason, the literature reports several different ways to couple CE to MS by a sheathless interface focusing on creating a distinct ESI electrical contact. Application of a conductive coating to the emitter tip, joining a conductive emitter tip to the CE capillary, insertion of a wire into the CE capillary end, and positioning a metal sleeve around a porous etched CE capillary wall are among the many propositions [162, 163]. Although sheathless interfaces present better detectability due to the absence of a sheath liquid, allow closer positioning of the CE capillary to the MS (increasing the effective analyte mass transfer), and exhibit improved ionization and droplet desolvation, it still poses some limitations. The disadvantages of sheathless CE-MS interfaces comprise: (i) The absence of commercially available apparatus (except for the recently launched interface based on the work of Moini, discussed below). (ii) EOF variation. (iii) Low robustness. (iv) Limited lifetime of the emitter tip. (v) Limited BGE composition selection, which must comprise volatile compounds, since the CE effluent is directly inserted into the MS system. Therefore, routine analysis with sheathless interfaces may be jeopardized because of the constant need for emitter tip substitution. In addition, low system repeatability is generally observed.

In 2007, Moini has shown for the first time a robust sheathless CE-MS interface, commercialized some years afterward by Sciex [164]. Nowadays, this is still the only sheathless interface that is commercialized with a CE-MS equipment. In Moini's design, the CE capillary tip has been etched with a 49 % HF solution (after removing the polyimide external coating) to obtain a porous tip to be inserted into the ESI needle, filled with BGE (Fig. 5.2c, d). The porous junction is necessary to allow ion transport for closing the CE electrical circuit and concomitantly to supply the ESI voltage. This interface has overcome the limitations imposed by the previously reported sheathless interfaces in many aspects: (i) Its fabrication is reproducible and automated. (ii) A single-step etching process makes the capillary tip porous and with a smaller outer diameter – the inner wall is preserved during fabrication by flowing nitrogen gas. (iii) Any tip disruption may be easily fixed by removing a small section of the capillary. (iv) Eventual electrolysis reactions occur outside the CE capillary, avoiding bubble formation, which would harm CE separation due to current interruption. Ramautar et al. have explored this interface configuration for profiling human urine metabolites [117].

A comparison of the performance of CE-ESI-MS sheath liquid and sheathless interfaces in terms of detectability for the analysis of intact proteins has been reported by Haselberg et al. [165]. Capillaries with a porous tip were inserted into a stainless steel needle filled with static conductive liquid and installed in a conventional ESI source. The same porous tip capillaries were used in a sheath liquid interface with isopropanol as SHL, resulting in fairly similar responses in terms of protein signals. However, limits of detection obtained with the sheath liquid interface were substantially higher than those obtained with the sheathless interface (from 82 to 136 times

higher), due to increased baseline noise levels in the former. Detection limits were overall improved by a factor of 6.5–20 with sheathless CE-MS.

5.2.3 CE-MS Methodologies for Metabolomics

CE-MS metabolomics studies are often conducted under electrospray ionization (ESI) with triple coaxial sheath flow interfaces and time-of-flight (TOF) mass analyzers [33–56]. Due to simplicity, CZE is the preferred CE mode in metabolomics, generating robust methods. Unlike LC-MS, the mobile phase or more precisely the BGE composition changes according to the selected ionization mode. The addition of low percentages of organic solvents to volatile BGEs is often sought to improve resolution. Baseline separation of leucine/isoleucine/*allo*-isoleucine isomers in methanol-modified formic acid BGE is a landmark [138, 145]. In addition, a sheath liquid that may be of distinct composition for each ionization mode is used to promote and/or enhance ionization at the ion source. Small cationic and anionic charged species are the expected metabolite targets visualized by CE separations. In CE-MS with positive ionization mode ($[M]^+$, $[M+H]^+$, $[M\text{-}H_2O+H]^+$, $[M+Na]^+$, etc.; M stands for molecule), it is possible to inspect amino acids, biogenic amines, and nucleosides, whereas the negative ionization mode ($[M\text{-}H]^-$, $[M+HCOO]^-$, $[2\ M+Na\text{-}2H]^-$, etc.) reveals carboxylic acids, phosphorylated carboxylic acids, phosphorylated saccharides, nucleotides, nicotinamide and flavin adenine coenzymes, as well as citrate isomers, dinucleotides, and CoA compounds [125].

A schematic representation of the overall possibilities CE-MS offers for the analysis of cationic and anionic metabolites is depicted in Fig. 5.3. Typically, cationic metabolites are screened in uncoated fused-silica capillaries with low pH volatile electrolytes, such as formic acid or acetic acid, generating a small but normal electroosmotic flow (EOF, flow toward the cathode due to little ionization of the capillary wall silanol groups). The CE system is operated under positive high voltage, applied at the capillary inlet, and it is connected to the MS via ESI in positive ionization mode (Fig. 5.3a). Anionic metabolites may also be screened in this format; however, high pH volatile electrolytes, such as ammonia/ammonium salt buffers (ammonium formate, acetate, or carbonate being the most commonly used), are mandatory to generate an EOF high enough to conduct the compounds which passed the interface toward the MS entrance. Nevertheless, since a positive voltage is applied at the capillary inlet, the anions will migrate counter-electroosmotically. Moreover, the ESI voltage needs to be set appropriately (negative ionization mode). This approach is not preferential for anionic metabolite analysis because by setting the migration of anions against EOF, long analytical runs are imposed and migration time repeatability might be compromised, which is already a concern in CE separations in bare fused-silica capillaries.

A more elegant solution for the CE-MS analysis of anionic metabolites is reached with the use of coated capillaries and high pH electrolytes (Fig. 5.3b, c). The CE system is now operated under a negative high voltage, applied at the capillary inlet,

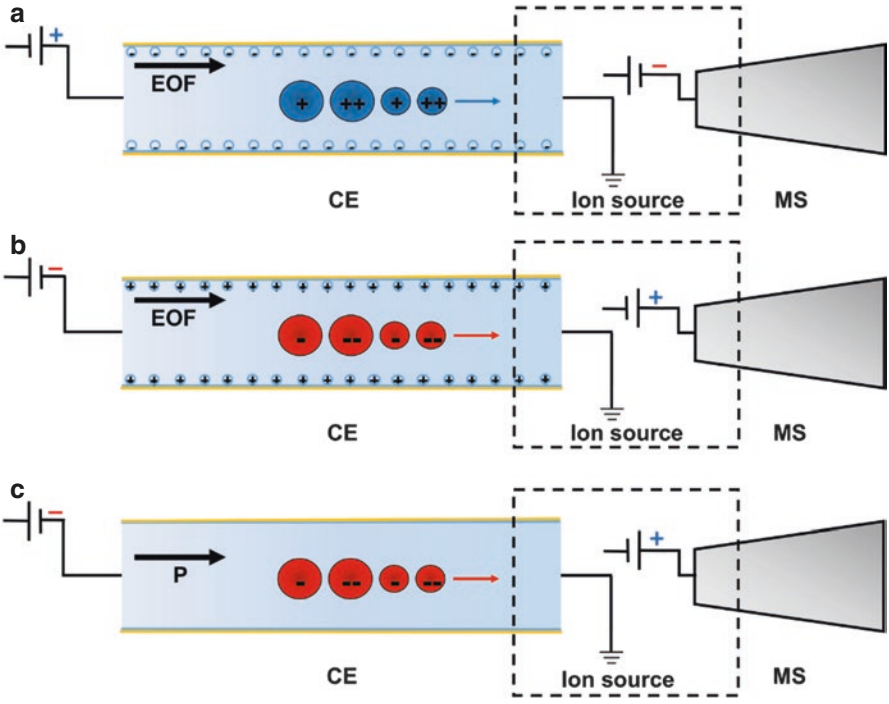

Fig. 5.3 CE-MS methodologies for untargeted metabolomics of cationic metabolites (**a**) and anionic metabolites using cationic polymer-coated capillaries (**b**) and neutral capillaries (**c**)

and it is connected to the MS via ESI in negative ionization mode. Either a cationic polymer coating (Fig. 5.3b) to reverse EOF (flow directed toward the anode) or a neutral coating (Fig. 5.3c) to eliminate EOF can be chosen. In the former, the negative species electrophoretic velocity and the electroosmotic flow velocity are in the same direction (toward the anode or positive pole), resulting in additionally faster separations. In the latter, since EOF is eliminated, a pressure-driven flow is usually implemented to prevent that sheath liquid components enter the separation capillary.

With these simple approaches, Soga and collaborators introduced untargeted metabolomics of biological cells using CE-MS platforms for the first time [125, 127]. By using all schemes of Fig. 5.3, a thorough evaluation of the metabolome of *Bacillus subtilis* cells upon the onset of sporulation was pursued. For the cationic metabolites screening, a BGE composed of 1 mol L^{-1} formic acid and a SHL comprised of 5 mmol L^{-1} ammonium acetate in 50 % methanol/water were used. Sets of 30 protonated $[M+H]^+$ ions were analyzed successively by SIM mode to cover the entire range of *m/z* from 70 to 1027 (Fig. 5.4). Anionic metabolites were screened in a BGE composed of 50 mmol L^{-1} ammonium acetate at pH 8.5 with a SHL comprised of 5 mmol L^{-1} ammonium acetate in 50 % methanol/water in a cationic polymer-coated capillary, SMILE(+). Nucleotides and coenzyme A compounds

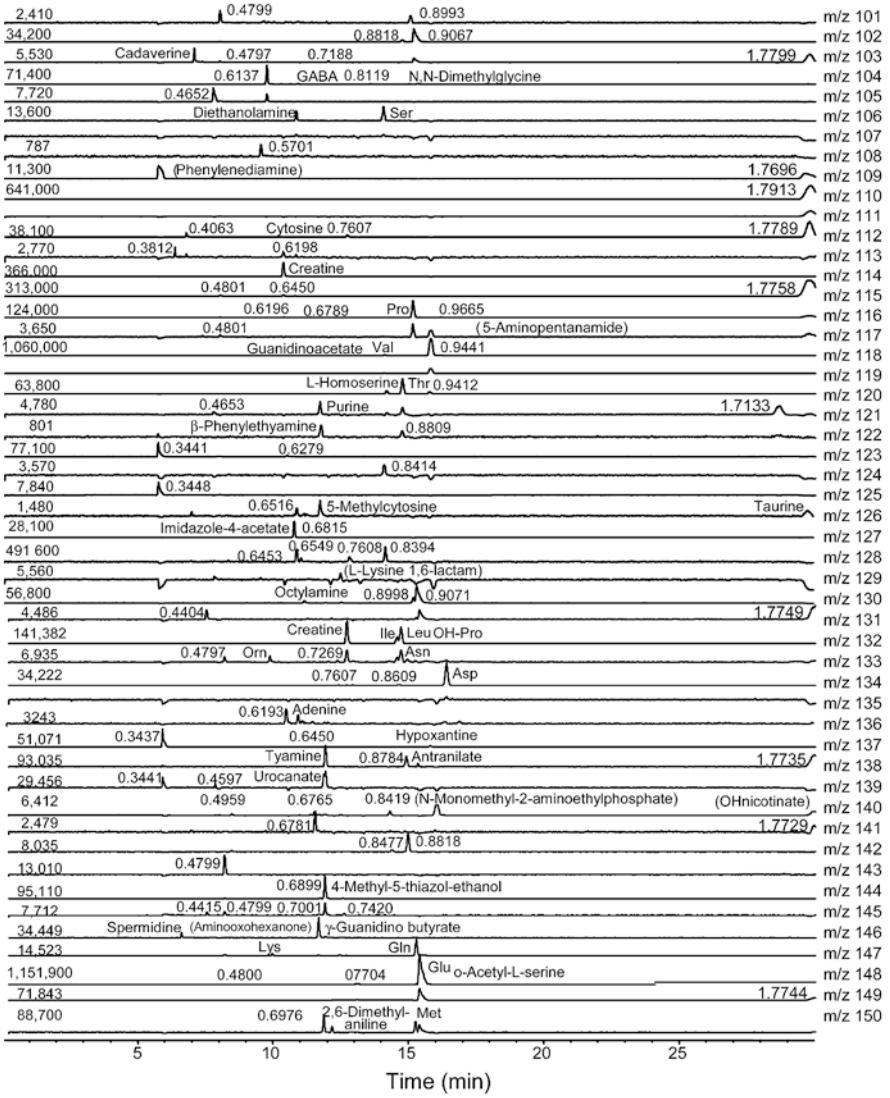

Fig. 5.4 Selected ion electropherograms for cationic metabolites of *Bacillus subtilis* in the range of *m/z*101–150. The numbers in the upper left corner of each trace are the abundances associated with the tallest peak in the electropherogram, for each *m/z*, and the numbers on top of peaks are relative migration times normalized with methionine sulfone (IS) (Reprinted with permission from Ref. [127])

were screened in a GC-coated capillary (polydimethylsiloxane, DB-1) in a slightly lower pH BGE, 50 mmol L^{-1} ammonium acetate at pH 7.5, with the same SHL. To prevent entrance of the SHL into the separation channel, a pressure of 50 mbar was applied to the capillary inlet promoting a flow of solution toward the anode. Exactly

1692 metabolites were catalogued, 150 were positively identified, and 83 were assigned based on the expected charge state and isotopic distribution. Later on, Soga and col. advocated the use of platinum ESI spray needle to replace stainless steel spray needles in the analysis of anionic metabolites [122]. It was observed that stainless steel was prone to oxidation and corrosion at the anodic electrode due to electrolysis; the resulting precipitation of iron oxides plugged the capillary outlet. Moreover, eventual complexation of anionic metabolites with iron and nickel ions generated by corrosion would reduce significantly detection sensitivity because the formed complexes are positively charged and move backward to the cathode (capillary inlet).

Within the context of CE-MS methodologies for metabolomics, a few parameters of interest will be examined. Because metabolomics studies are comparative in nature, they demand high-precision measurements. Migration time repeatability, essential in untargeted metabolomics studies, and/or peak area repeatability, crucial in targeted metabolomics quantitation studies, must be addressed properly. It is well accepted that migration time variability is a consequence of EOF variability, which in turn is related to the capillary inner surface state and integrity. Thus, adsorption of solutes to the capillary wall and/or any sort of adverse solute-wall interactions, electrolyte components-wall interactions, etc. will compromise the EOF magnitude and consequently affect migration time repeatability. Many authors have addressed this issue by using covalently coated capillaries, such as the work of Soga and col. referred above [122, 125, 127]. De Jong and collaborators have proposed to modify the capillary walls dynamically with charged polymers [166]. Bilayers constituted of polybrene (PB) and poly(vinyl sulfonate) (PVS) or triple layers constituted of PB, dextran sulfate (DS), and PB have been extensively investigated to the metabolic profiling of biofluids [113, 114, 144]. Overall, covalently bound polymers are still preferred in metabolomics studies due to stability and durability. Moreover, any leakage of polymer during CE operation cannot be tolerated, especially if it results in contamination of the mass analyzer.

Full coverage of metabolites by any hyphenated technique to MS demands the use of both positive and negative ionization modes. It is worth mentioning that a large fraction of metabolites in biological fluids is acidic in nature and can only be ionized efficiently using negative ionization. However, signal-to-noise ratios in negative ionization mode are often low by two to three orders of magnitude when compared to positive ionization, thereby limiting sensitivity in metabolomics applications [112]. Reduced MS signals for anions have been attributed to analyte ionization suppression by the presence of acetate ions in the BGE and/or SHL [114, 167]. To circumvent this loss of sensitivity, the transformation of anionic metabolites into cationic compounds by derivatization or complexation has been proposed, allowing positive ionization mode to be applied [167, 168]. With these methodologies, sensitivity indeed improved for anionic compounds, and more favorable detection limits were achieved. However, derivatization procedures increase sample pretreatment complexity, and losses of metabolites can occur due to incomplete derivatization. Furthermore, not every anionic compound can be derivatized efficiently. Therefore, a great deal of development is still necessary for

the CE-MS analysis of the metabolome in negative ionization mode, despite the efforts toward the testing of new BGE additives [112].

Still regarding ionization modes, an alternative strategy to simplify procedures during data acquisition in CE-MS metabolomics has been proposed by Gulersonmez et al. [132]. A single BGE at an intermediary pH is used for both positive and negative ionization modes, e.g., pH 3.0 acetic acid. This pH is low enough to protonate most of the metabolites exhibiting basic moieties (biogenic amines, amino acids, etc.) generating cationic compounds, and at the same time, it is high enough to promote partial dissociation of those metabolites with acidic moieties (carboxylic acids, nucleotides, etc.) generating anionic compounds. Therefore, the same BGE is used to screen both cationic and anionic portions of the metabolome, in consecutive runs (TOF mass analyzers), by a simple switch of the ESI voltage.

Analytical frequency is another parameter of concern in metabolomics studies, since runs are usually long to ensure that a large variety of metabolites of differing properties is inspected. Multiple sequential injections of samples (volumetric transfer of sample to the separation capillary by applying pressure at capillary inlet), intercalated by injections of BGE zones, before the high voltage has been set, are a classical CE strategy to improve analytical frequency, and it has been implemented advantageously in CE-MS metabolomics by Britz-McKibbin and collaborators [140]. The authors developed a multi-segment injection (MSI) as a multiplexed CE-MS platform in which a serial injection of seven or more discrete human plasma sample segments could be performed within a single capillary without compromising the separation quality and/or quantitative performance. The overall MSI scheme is depicted in Fig. 5.5 and increased sample throughput by one order of magnitude. By using a seven-segment sample injection for single-step acquisition, building of external and processed analytical curves for quantitation of polar metabolites and isomers in plasma, with acceptable accuracy and precision, use and/or selection of internal standards, running recovery tests samples, identification strategies via pattern recognition, etc., have all been successfully demonstrated.

5.3 Representative Applications of CE-MS in Clinical Metabolomics

Capillary electrophoresis as an analytical platform to assess metabolites in biological samples has been used for decades. The work of Jellum and collaborators in the profiling of organic acids in biofluids, using UV detectors to screen metabolic diseases, may be referred as the first CE-based clinical metabolomics [169–171]. By the same token, the work of Barbas and collaborators, who investigated metabolic disorders, known as inborn errors of metabolism, by screening short-chain carboxylic acids in human urine, is another innovatory example of the diagnostic power of CE technology [172, 173]. The pioneerism of global metabolic fingerprinting or untargeted metabolomics using CE-MS platforms has been attributed unequivocally to Soga's research group [125, 127], as described previously in this chapter and summarized by the

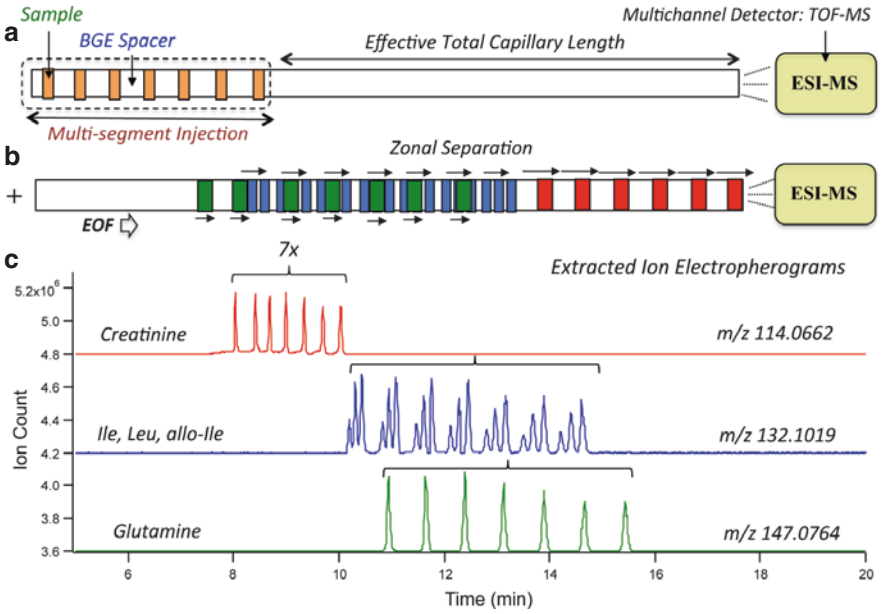

Fig. 5.5 Multiplexed separation based on serial injection of seven discrete sample segments within a single capillary by MSI-CE-MS (**a**), where (**b**) ions migrate as a series of zones in free solution prior to ionization. This format enables reliable quantification of polar metabolites and their isomers in different samples since ionization occurs within a short time interval (≈2–6 min) under steady-state conditions when using a high mass resolution TOF-MS (**c**) (Reprinted with permission from Ref. [140])

group reviews [36, 174]. At this point, it is worth mentioning the important contribution of Mishak's research group, who has established the reliability of CE-MS platforms for the initial diagnosis and prognosis of the progression of numerous diseases via biomarker discovery by mapping endogenous peptides in human urine [175, 176].

Table 5.1 compiles many examples of CE-MS in clinical metabolomics organized by studied condition or disease, biological matrix, metabolomics approach, and type of metabolites screened (targeted metabolomics) or revealed (untargeted metabolomics). A few details of the analytical methods, such as BGE and/or SHL composition, type of mass analyzer, sample preparation procedures, algorithm and/or software used in data treatment, and finally whether the study was exclusively conducted by CE-MS or data was acquired in a multiplatform setup, were also provided.

Table 5.1 was meant to present a comprehensive revision of the literature in the period from 2001 to 2016. Based on the relatively small number of applications Table 5.1 brings, associated with an even smaller number of groups researching in the field, it is fair to conclude that CE-MS in the clinical metabolomics scenario has still much room for growth. A possible explanation for the rather limited use of CE-MS technology in this field might be related to the fact that CE-MS is still considered a novelty compared to other much more established techniques, such as GC-MS, LC-MS, and NMR, and there is a certain resistance to consider its use in metabolomics. Issues such as migration time variability, sample loadability and throughput,

low concentration sensitivity, etc. are still of concern by many metabolomics leading groups, despite the relevant advancements made over decades to overcome CE-MS technical and methodological difficulties, as thoroughly discussed in this chapter. Another relevant aspect that might hinder the use of CE-MS in clinical metabolomics studies is the lack of standardized operating protocols. As Table 5.1 contents sustain, each research group develops and implements its own method, with small but tangible variations of capillary coatings, dimensions and conditioning, BGE and/or SHL composition, as well as MS type and parameters. There is no convergence toward a single optimized strategy to perform untargeted metabolomics studies, for instance, or a complete detailed protocol for metabolomics, as it is the case for GC-MS-, LC-MS-, and NMR-based metabolomics. Such protocol would boost sales of CE-MS instrumentation, allowing the creation of an universal database for metabolite identification, and stimulate applications in clinical metabolomics, among other areas.

Metabolomics studies follow a general workflow, comprising problem formulation, experimental design, sample preparation, data acquisition and processing, statistical analysis, metabolite identification, association to metabolic pathways, and biological validation. All these steps were critically discussed in Chap. 1. Notably, a large number of applications compiled in Table 5.1 were conducted under the premise of untargeted clinical metabolomics, i.e., to improve the knowledge on the onset and progression of a given disease at metabolic level and to search discriminant metabolites that could be used further on for diagnosis and/or prognosis purposes. Furthermore, most of the reported CE-MS methods in Table 5.1 were conducted with high-resolution mass spectrometers using sheath liquid interfaces (available commercially much longer than sheathless interfaces), using low pH BGE and aqueous methanolic SHL. Untargeted metabolomics studies comprise simpler sample treatments, involving protein precipitation, followed by filtration and dilution. Sample procedures for targeted metabolomics are of course dependent on the identity of the metabolites under investigation. Overall sample preparation strategies for metabolomics and their impact on results have been revised by many authors [42, 106, 177, 178]. Quality control samples (QC), pool of all control and test samples under consideration in a given study, have often been considered to attest platform stability during data acquisition. Method validation concepts for untargeted metabolomics have also been reviewed [157].

Another feature of Table 5.1 is that data preprocessing often relies on in-house developed algorithms or free access softwares with PCA, PLS-DA, and OPLS-DA being the preferred multivariate data analysis. Peak alignment in such algorithms is usually of great importance because it deals with the intrinsic migration time variability of CE-MS data and it has long been a topic of investigation [179–182]. A great challenge in metabolomics studies in general is metabolite identification. In CE-MS platforms, several authors have demonstrated strategies of peak identification using mobility in conjunction with accurate m/z values [183–185]. Finally, analytical multiplatform studies in Table 5.1 are rare.

An illustration of the contents of Table 5.1, discriminating conditions and diseases studied so far by CE-MS under the metabolomics perspective, is depicted in Fig. 5.6a. It is readily observed that several types of cancer received a great deal of attention by the scientific community. Fig. 5.6b shows the temporal evolution of publications in the field denoting the growing interest CE-MS has drawn in the last decades.

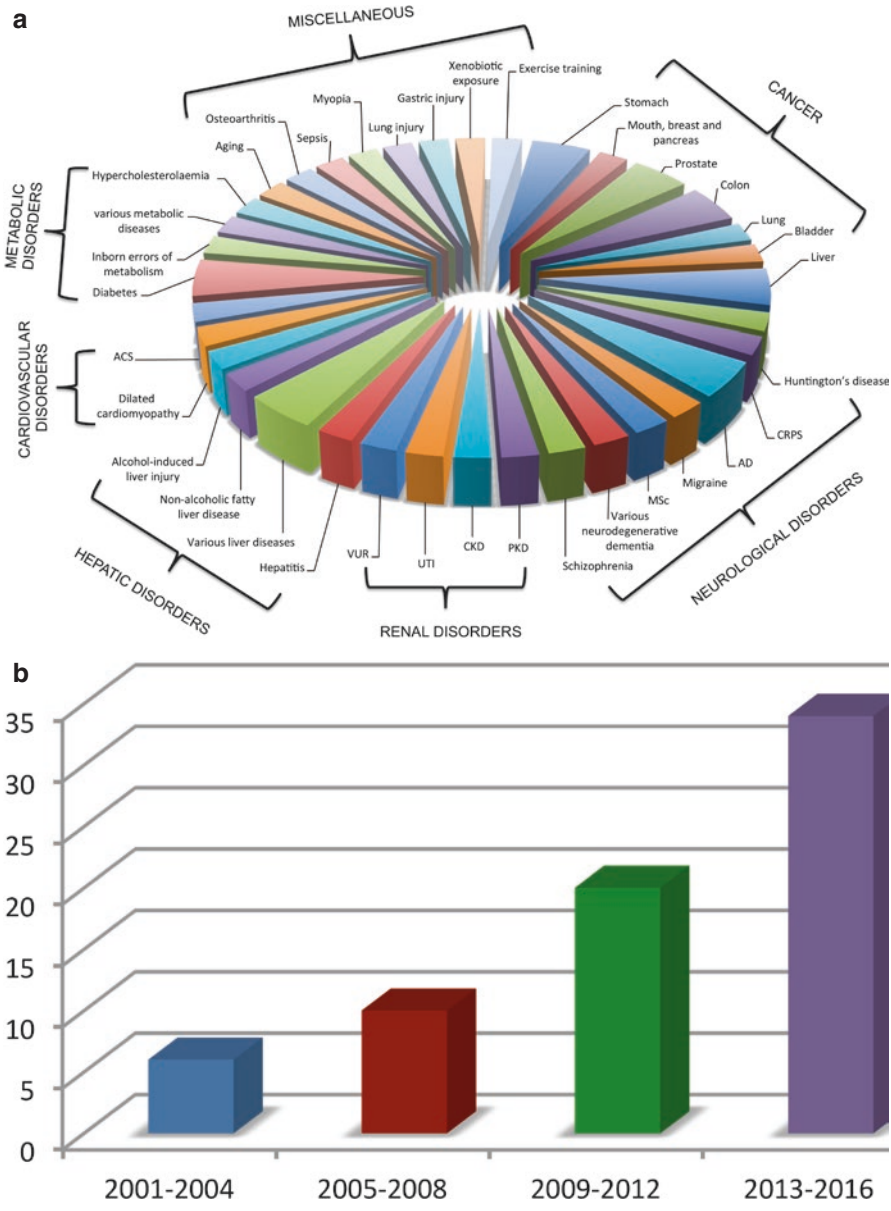

Fig. 5.6 CE-MS in clinical metabolomics. Literature publications compiled in Table 5.1 were organized by type of condition and/or disease (**a**) and temporal progression of articles (**b**). Legends: *ACS* acute coronary syndrome, *AD* Alzheimer's disease, *CKD* chronic kidney disease, *CRPS* complex regional pain syndrome, *MSc* multiple sclerosis, *PKD* polycystic kidney disease, *VUR* vesicoureteral reflux, *UTI* urinary tract infection

5.4 Conclusions and Perspectives

From its inception in the late 1980s, CE-MS has matured into a resourceful technique that encompasses the analysis of compounds from many different chemical classes, especially those with ionic and/or highly polar character that constitute an important subset of the human metabolome. Relevant features of CE, such as high efficiency and resolution power, fast analysis time, multiple separation modes, use of aqueous-based electrolytes, compatibility with biofluids, small sample volume, etc. were combined with the remarkable detection sensitivity, extra selectivity, and spectral information provided by MS technologies. Several technical difficulties related to early CE-MS interface designs and platform stability issues have been tackled and improved considerably, especially in the last decade that was testimony to the commercial launching of complete CE-MS systems. Intrinsic aspects related to the technique performance have also been addressed properly, allowing CE-MS to grow into a robust technology for metabolomics.

Despite the clear adequacy of CE-MS for clinical metabolomics and the technical improvements evidenced over the years, the field is still underrepresented when compared to the contribution of other well-established NMR and chromatography-based platforms, showing a rather limited number of research leading groups actively working in the area. Implementation of analytical multiplatform approaches, necessary to establish a more comprehensive coverage of the metabolome, analysis of larger clinical cohorts, expansion of the applicability to key diseases and conditions, and setting up interlaboratorial validation studies are a few strategies that should boost the use of CE-MS in clinical metabolomics and build user confidence in the technology.

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