Title	A reflection on two decades of metabolomic innovations in capillary electrophoresis-mass spectrometry at the Institute for Advanced Biosciences, Keio University
Sub Title	メタボロミクスの技術革新に対する慶應義塾大学先端生命科学研究所の20年に亘る貢献
Author	Britz-McKibbin, Philip
Publisher	慶應SFC学会
Publication year	2023
Jtitle	Keio SFC journal Vol.22, No.2 (2022.) ,p.24- 41
JaLC DOI	10.14991/003.00220002-0024
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Notes	特集 SFCバイオの軌跡 第1章 先端生命科学 招待論文 : 総説・ レビュー論文
Genre	Journal Article
URL	https://koara.lib.keio.ac.jp/xoonips/modules/xoonips/detail.php?koara_id=AA11671240-00220002-0024

［招待論文：総説・レビュー論文］

A Reflection on Two Decades of Metabolomic Innovations in Capillary Electrophoresis-Mass Spectrometry at the Institute for Advanced Biosciences, Keio University

メタボロミクスの技術革新に対する慶應義塾大学先端生命科学研究所の 20 年に亘る貢献

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Abstract: The Institute for Advanced Biosciences (IAB) at Keio University was first established in April 2001 to become a leading international metabolomics center for advancing integrated systems biology research. Unlike other metabolomic centers worldwide, IAB strategically focused on developing capillary electrophoresis-mass spectrometry (CE-MS) technology as their central instrumental platform for comprehensive analysis of polar/ionic metabolites in conjunction with bioinformatic tools for biomarker discovery and biochemical interpretation. In addition to hosting two International Metabolomics Society conferences, training young scientists and founding successful spin-off companies, IAB has contributed to high impact metabolomic studies as applied to clinical medicine, functional genomics and multi-omic studies, cancer diagnostic testing, agriculture and food science, as well as epidemiology and population health. IAB has also fostered metabolomic partnerships with the local community, clinicians and industry as reflected by their involvement in a prospective study of community-dwelling residents from Tsuruoka City and assessment of the quality of foods produced from the Shonai area. IAB has also spearheaded numerous CE-MS based metabolomic research collaborations elsewhere across Japan and worldwide. This short review will strive to highlight key milestones spanning over 20 years of research innovations in CE-MS metabolomics technology brought forth under the leadership of Dr. Masaru Tomita.

慶應義塾大学先端生命科学研究所 (IAB) は 2001 年 4 月に設立され、統合システム生物学研究を推進するための国際的なメタボロミクスセンターとしての役割を果たしている。世界中の他のメタボロミクスセンターとは異なり、IAB は極性・イオン性代謝物の包括的な解析プラットフォームとしてのキャピラリー電気泳動質量分析（CE-MS）技術の開発と、バイオマーカー探索や生化学的解釈のためのバイオインフォマティックツールの開発に重点を置いて戦略的に行ってきた。IAB は 2 回の国際メタボロミクス会議の主催、若手科学者のトレーニングやスピンオフ企業の設立に加えて、臨床医学、機能ゲノミクス、マルチオミクス研究、がん診断検査、農業および食品化学、疫学や公衆衛生学等の、インパクトの大きなメタボロミクス研究に貢献してきた。IAB はまた、鶴岡市の地域住民を対象とした前向き研究の実施や庄内地域で生産された食品の品質評価に代表されるように、地域社会、臨床医や産業界とのメタボローム研究を介したパートナーシップを育んできた。さらに、IAB は日本および世界の数々の CE-MS を用いたメタボローム共同研究の先頭に立ってきた。この短いレビューでは、冨田勝博士のリーダーシップの下でもたらされた 20 年以上に亘る CE-MS メタボロミクス技術の研究イノベーションの主要な成果について紹介する。

Keywords: metabolomics, capillary electrophoresis-mass spectrometry (CE-MS), non-targeted metabolite profiling, biomarker discovery, clinical medicine
メタボロミクス、キャピラリー電気泳動ー質量分析（CE-MS）、非標的代謝物プロファイリング、バイオマーカー探索、臨床医学

1　Establishing CE-MS as a Robust Platform for Metabolomics: The Early Years

The principles of biochemical individuality and disease susceptibility (Garrod, 1902; Williams, 1956) may be revealed via quantitative metabolite profiling (Horning & Horning, 1971; Pauling et al., 1971), which can be traced back over the past century. However, a renaissance in metabolism was sparked more than twenty years ago when metabolomics was proposed as a functional genomics tool to understand the role of gene mutations on molecular phenotype with the advent of large-scale genomic sequencing projects (Oliver et al., 1998; Fiehn et al., 2000). Contemporary metabolomics research has relied on recent advances in analytical instrumentation and computational analysis, including high-resolution mass spectrometry (MS) and high-field nuclear magnetic resonance (NMR) infrastructure (Kuehnbaum & Britz-McKibbin, 2013). For instance, gas chromatography-mass spectrometry (GC-MS)

and increasingly liquid chromatography-mass spectrometry (LC-MS) using reversed-phase and/or hydrophilic interaction modes of separation are widely used in metabolomic studies. Alternatively, capillary electrophoresis-mass spectrometry (CE-MS) offers a high efficiency microseparation platform ideal for characterization of the ionic metabolome from volume- or mass-limited biospecimens, such as residual infant sweat (Nori de Macedo et al., 2017), a single dried blood spot punch (DiBattista et al., 2019), or a lyophilized muscle tissue biopsy (Saoi et al., 2019). Dr. Masaru Tomita early on recognized the potential of CE-MS technology for metabolomics since the majority of primary metabolites within cells are hydrophilic/charged ions, whose measurements could be used to simulate cell behavior *in silico* for deeper insights into dynamic metabolic processes critical to life (Tomita et al., 1999).

In 2003, researchers from IAB at Keio University first reported a methodology for untargeted metabolite profiling using CE-MS allowing for determination of 352 metabolite standards, along with characterization of a total of 1692 known and unknown metabolites from *B. subtilis* extracts (Soga et al., 2003). In this case, a coaxial sheath liquid interface was used to couple CE with a time-of-flight (TOF) mass spectrometer, which was operated initially under three distinct modes as depicted in **Figure 1**. This included an acidic buffer (1.0 M formic acid, pH 1.8) for separation of cationic metabolites in an unmodified fused-silica capillary under positive ion mode, an alkaline buffer (50 mM ammonium acetate, pH 8.5) for separation anionic metabolites using a cationic polymer coated capillary under negative ion mode, and a second alkaline buffer (50 mM ammonium acetate, pH 7.5) for resolving highly charged nucleotides and coenzyme A metabolites with a neutral coated capillary under negative ion mode. The latter pressure-assisted CE-MS mode was deemed necessary due to deleterious adsorption of multivalent anions to the cationic polymer coated capillary (Soga et al., 2002a) as electrophoretic separations were performed under reversed polarity, such that anions co-migrate with an anodic electroosmotic flow (EOF) to avoid deleterious current drops (Soga et al., 2002b).

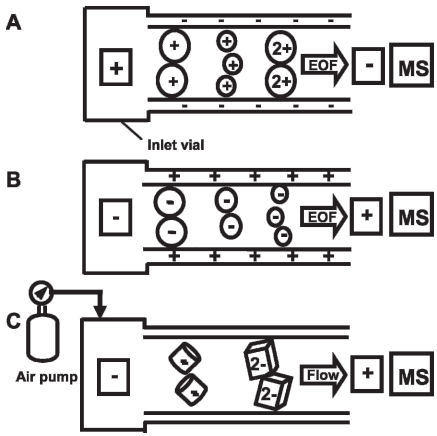

Figure 1. Original CE-MS protocol used for comprehensive metabolite profiling of *B. subtilis* cell extracts performed under three different configurations for the analysis of (A) cationic metabolites, (B) anionic metabolites, and (C) multivalent anions, such as nucleotides and coenzyme A metabolites (Reproduced from Soga et al., 2003).

Pressure assisted-CE-MS for analyzing multivalent nucleotides was later proposed using a silanol masking technique by preconditioning the cationic polymer coated capillary with phosphate buffer prior with the nebulizer gas turned off to avoid ion suppression (Soga et al., 2007). However, this approach was complicated and time consuming since repeat preconditioning with an involatile phosphate buffer was needed prior to each run to obtain adequate reproducibility.

A major breakthrough to improve the long-term stability of CE-MS for anionic metabolite profiling under reversed polarity was discovered when using a platinum electrospray ionization spray needle (Soga et al., 2009) that addressed the poor robustness reported in an inter-method comparison of cellular metabolites by CE-MS relative to LC-MS and GC-MS protocols (Büscher et al., 2009). This problem was subsequently attributed to incidental corrosion of the stainless steel sprayer in CE-MS at the anodic electrode due to electrolysis that eventually plugged the capillary outlet with iron oxide precipitates resulting in shortened capillary lifetimes (Soga et al., 2009) as shown in **Figure 2**. This new method had several advantages since it integrated two CE-MS previous methods originally developed for anionic metabolites under negative ion mode (**Figure 1B,C**) into a single robust protocol that also

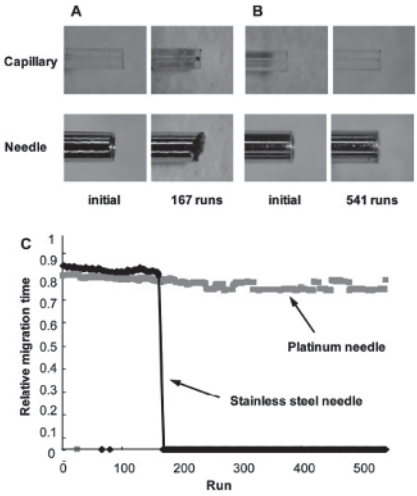

Figure 2. Improved long-term robustness of CE-MS protocol with a coaxial sheath liquid sprayer for anionic metabolites with a cationic polymer coated capillary under reversed polarity when using a (A) standard stainless steel and a (B) platinum needle. The greater endurance and reproducibility of CE-MS (C) with the platinum as compared to stainless steel needle are evident after 541 repeated sample injections were achieved as it avoids needle corrosion and plugging of the capillary outlet by iron oxides due to electrolysis (Reproduced from Soga et al., 2009).

improved sensitivity for certain metal chelating anions (e.g., citrate). As a result, two CE-MS protocols were standardized for cationic and anionic metabolite profiling, which were implemented into future metabolomic studies at IAB. This example highlights the importance of rigorous method optimization and validation studies when developing reliable CE-MS methods since other operating conditions may impact anionic metabolite profiling under negative ion mode (van Mever et al., 2019). For instance, aminolysis of the polyimide coating of the fused-silica capillary can occur when using strongly alkaline ammonium based volatile buffers (pH > 9.0), which leads to incidental capillary fractures with shorter lifetimes (Yamamoto et al., 2016).

A major bottleneck when performing nontargeted metabolomics involves pre-processing of raw data files in CE-MS, which requires multiple steps for spectral deconvolution, data filtering, data smoothing, and peak picking (Shanmuganathan et al., 2021b). Indeed, there exists a large number of poorly characterized peaks in untargeted metabolomics using ESI-MS, including unreliable and redundant signals generated from the same compound (Saito et al., 2021). Although there are several

open-source and vendor-independent software programs optimized for LC-MS metabolomics (e.g., XCMS, mzMine), the distinct electromigration behavior and peak shapes for ions in CE-MS may benefit from customized solutions than existing vendor software developed for chromatographic separations. For these reasons, researchers at IAB first developed a Mathematica software package referred to as MathDAMP for differential analysis of metabolomic data sets to facilitate their direct comparisons for biomarker identification (e.g., disease versus control). Raw data sets were automatically preprocessed and normalized in terms of migration times and integrated peak areas and then visualized using density plots (Baran et al., 2006). Later, Morohashi et al. (2007) introduced a post-measurement peak filtering method for CE-MS, referred to as P-BOSS, as a way to prevent data overfitting due to a large fraction of spurious signals and background peaks detected in nontargeted metabolomic data workflows. A proprietary software referred to as MasterHands was later developed for processing and annotating raw data generated by CE-TOF-MS (Sugimoto et al., 2010b). Furthermore, researchers at IAB have also contributed to a widely used KEGG-based pathway visualization tool for complex -omics data sets (Arakawa et al., 2005), including a web-based metabolite pathway browser to aide in the biochemical interpretation based on the KEGG Atlas (Kono et al., 2009). As a result, Dr. Masaru Tomita commanded several important milestones in CE-MS for robust methodology, software development and bioinformatic tools to enable cutting-edge metabolomics research.

2　Novel Developments in CE-MS for Improving Sensitivity and Unknown Identification

Concentration sensitivity remains a major limitation in CE-MS based metabolomics as compared to LC-MS due to the small injection volumes introduced on-capillary (~ 10 nL) when using a conventional coaxial sheath liquid interface as it leads to post-capillary dilution effects while stabilizing spray formation (Maxwell & Chen, 2008). In this case, a sheathless CE-MS interface based on a porous sprayer

design was evaluated for the analysis of cationic metabolites (Hirayama et al., 2012), which was found to improve the detection limit for a subset of compounds by more than 5-fold corresponding to a 10-fold increase metabolome coverage of urine samples relative to a sheath flow CE-MS. However, a longstanding obstacle has been the long-term stability and reliability of various sheathless CE-MS interface designs that may be too fragile and expensive to routinely operate. Researchers at IAB later developed their own customized sheathless CE-MS interface easily fabricated by making a small crack about 2 cm from the end of a capillary column, which was fixed on a plastic plate with a dialysis membrane to prevent sample loss (Hirayama et al., 2018). Despite its convenience and cost-effectiveness, only a modest sensitivity gain of 2.5-fold was achieved for cationic metabolites analyzed from cancer cell extracts as compared to a conventional sheath liquid CE-MS system. In contrast, sheathless CE-MS together with on-line sample preconcentration via transient isotachophoresis has been reported to greatly improve concentration sensitivity by two orders of magnitude allowing for deeper urine metabolome coverage (Ramautar et al., 2012).

To date, nontargeted CE-MS metabolomic analyses have been largely performed using TOF mass analyzers due to their high mass resolution (~ 40,000 full-width at half maximum or FWHM), fast acquisition times, and favorable cost-benefit analytical performance. Recently, a new electrospray ionization adapter was developed to couple CE with a commercial Orbitrap mass analyzer, which offers greater mass resolution (~ 140,000 FHWM) and better sensitivity for detection of lower abundance metabolites than a conventional TOF system as depicted in **Figure 3** (Sasaki et al., 2019). For the first time, CE with ultra-high resolution MS using a sheath flow interface with grounded nebulizer was used to quantify over 270 cationic and anionic metabolites in plasma filtrate extracts with good linearity, accuracy and repeatability following method validation. Importantly, a large number of bioactive plasma peptides and their isobars were identified and resolved by this platform that have not been previously reported. Future studies are needed to better assess the long-

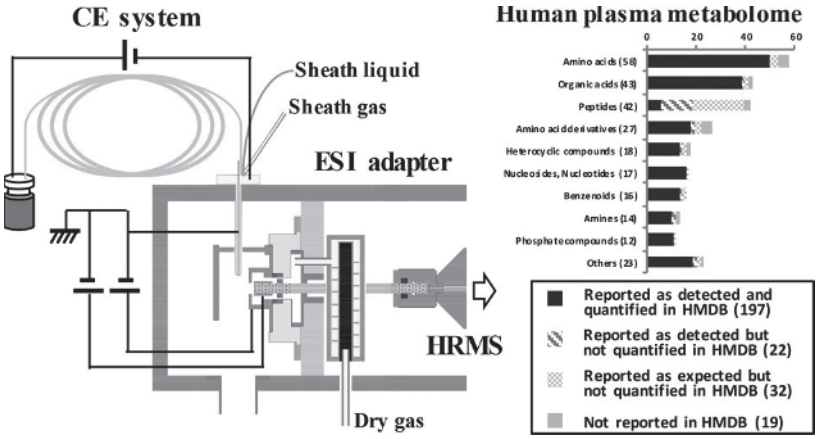

Figure 3. Deeper plasma metabolome coverage by CE-MS was achieved when using a customized sheath flow and grounded nebulizer interface as an electrospray ionization adaptor when coupled to an ultrahigh resolution orbitrap MS system (Reproduced from Sasaki et al., 2019).

term performance of this new CE-MS platform while developing integrated software control to improve instrument usability in metabolomic studies. Improved concentration sensitivity can be also achieved in CE when using multiple reaction monitoring with tandem mass spectrometry (MS/MS) that is optimal for targeted metabolite analysis of specific metabolite classes, as well as clinical and exposure biomarkers with lower detection limits, such as amino acids (Soga et al., 2004), γ-glutamyl dipeptides (Saoi et al., 2020) and 1-hydroxypyrene glucuronide (Gill & Britz-McKibbin, 2020).

Apparent migration times of ions in CE are more variable than retention times in reversed-phase LC due to changes in the EOF between runs, which can result in erroneous peak alignment and mis-annotation of metabolites from complex mixtures (Salzer et al., 2022). On the other hand, the effective (or apparent) electrophoretic mobility represents a more reliable and fundamental physicochemical property of an ion that can also support unknown metabolite identification when using computer simulations complementary to collision-induced dissociation tandem mass

spectrometry (MS/MS) (Lee et al., 2007). In fact, a recent inter-laboratory ring trial of a standardized CE-MS method for analysis of 21 cationic metabolites using different instrumental configurations across 13 laboratories demonstrated acceptable reproducibility when measuring the effective electrophoretic mobility of an ion rather than its relative migration time normalized to a single internal standard (Drouin et al., 2020). As a result, Sugimoto et al. (2005) proposed the use of two internal standard to better normalize apparent migration times for cationic metabolites in CE-MS in support of their identification among several putative isomeric and isobaric candidates. In this case, the electromigration behavior of ions was accurately predicted when using an artificial neural network algorithm based on their *pKa* (since it reflects the effective charge of an ion) as well as 152 other molecular descriptors derived *in silico* from their known chemical structure. This approach correctly classified metabolites in 78% of cases among the top three candidate ions following a Kyoto Encyclopedia of Gene and Genomics (KEGG) and Human Metabolome Database (HMDB) search (Sugimoto et al., 2005). Researchers at IAB later refined this strategy for unknown metabolite identification in CE when modeling the normalized migration time of 375 cationic metabolites as training set while using support vector regression to prevent model overfitting (Sugimoto et al., 2010a). This method was then used to predict the normalized migration times for 2,938 polar metabolites in CE-MS, which improved the identification rate for peaks assigned to likely candidate ions from open-access public databases. Future work may also be extended towards virtual quantification of recently identified metabolites when using CE when standards are unavailable based on modeling of solute ionization responses in ESI-MS that can vary widely for equimolar concentrations of metabolites in solution (Chalcraft et al., 2009).

3　CE-MS for Large-scale Metabolomics Studies: Beyond Polar Metabolites?

To date, there have been sparse large-scale CE-MS metabolomic studies given

lingering concerns of long-term instrumental stability and robustness over several years of prospective laboratory analyses (Belczacka et al., 2018; Shanmuganathan et al., 2021b). Researchers at IAB have been involved with the Tsuruoka Metabolomics Cohort Study (TMCS) comprising the recruitment of 11,002 local Japanese participants aged from 35 to 74 years, which may provide new insights in dietary exposures and lifestyle on disease risk assessment (Sasaki et al., 2018). For instance, Harada et al. (2016) first reported the analysis of 115 polar metabolites from fasting plasma samples using standardized CE-MS methods, which were collected from 896 Japanese men who participated in the baseline survey of the TMCS. In this work, nineteen metabolites were associated with daily alcohol consumption which was validated in an independent replication population, whereas three plasma metabolites were associated with alcohol induced liver injury, namely threonine, glutamine, and guanidinosuccinate (Harada et al., 2016). A follow-up study also identified plasma biomarkers associated with physical inactivity from 1,193 participants in the TMCS cohort when using CE-MS as it is a modifiable risk factor that increases chronic disease burden (Fukai et al., 2016). A panel of plasma metabolites, including several amino acids and their derivatives were associated with self-reports of sedentary behavior when using linear regression models with covariate adjustments, including age, body mass index, smoking, alcohol intake, and energy intake. The same group subsequently reported the absolute concentrations of 94 polar metabolites in 8,413 fasting plasma samples from participants in the TMCS study, which demonstrated good intermediate precision with acceptable intra-batch and inter-batch variability (Harada et al., 2018). To accomplish this milestone, four dedicated CE-TOF-MS instruments were used for metabolomics analyses with data acquired over 52 months (30 samples per day with QC every 10 runs) comprising 105 running batches of cations and 99 batches of anions. An inter-laboratory method comparison was also performed for plasma creatinine and uric acid concentrations measured by CE-MS as compared to validated clinical enzyme assays on matching serum samples, which demonstrated good mutual agreement with a mean bias of 10 to 13% (Harada et al.,

2018). A non-targeted approach was later applied to classify food intake biomarkers in plasma from 7,012 eligible participants from the TMSC study who completed a standardized food frequency questionnaire (Shibutami et al., 2021). Overall, 21 plasma metabolites were associated with the intake of nine self-reported food groups, such as hydroxyproline for meat, trimethylamine-N-oxide for fish, choline for eggs, as well as quinic acid and trigonelline for coffee. A large-scale CE-MS urine metabolomic study was also recently reported for 6,720 participants from the TMCS cohort, which included the quantification of 123 polar metabolites (Ishibashi et al., 2021). This study highlighted that spot morning urine samples are suitable for epidemiological studies given their consistent correlation with metabolites measured in a sub-set of matching 24-h urine samples with acceptable reproducibility for most compounds as reflected by a CV < 20% measured intermittently from QC samples.

Given the lower throughput of electrophoretic and chromatographic separations that rely on single sample injections with column reconditioning (< 30-40 min/sample), serial sample injections based on multisegment injection (MSI)-CE-MS offer a simple way to greatly boost sample throughput, encode spectral information temporally while also ensuring better long-term data fidelity without added infrastructure costs (Kuehnbaum et al., 2013), including a robust QC-based batch correction for longitudinal metabolomics data (Shanmuganathan et al., 2021a). This renders MSI-CE-MS competitive with robust NMR metabolomic methods given its greater sensitivity and metabolome coverage, smaller sample volume requirements, as well as lower operating costs and greater sample throughput (Shanmuganathan et al., 2021a). Recently, Igarashi et al. (2021) demonstrated that up to 40 consecutive samples can be analyzed by MSI-CE-MS in a single run with an effective sample throughput of 1 min/sample with acceptable reproducibility and accuracy when using matching deuterated internal standards for correction of ion suppression effects as shown in **Figure 4**. This approach was applied to a targeted analysis of salivary polyamines when using CE-MS/MS that found N1-acetylspermine as a promising biomarker for colorectal cancer. Moreover, there was a strong correlation in salivary

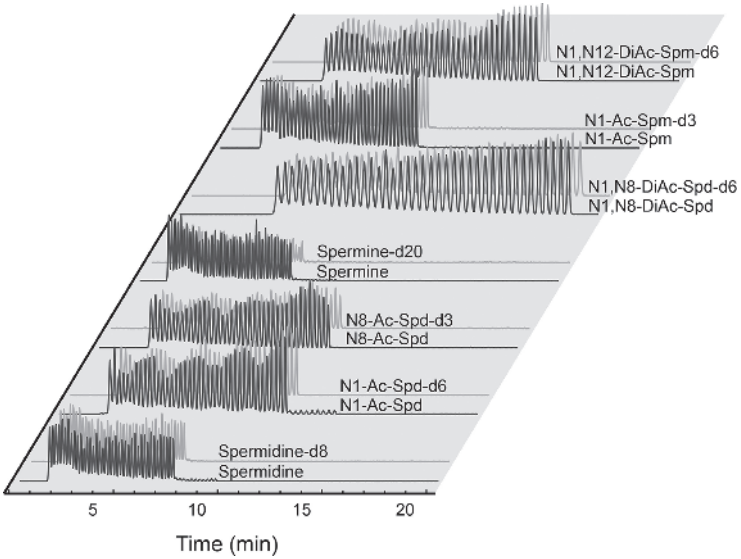

Figure 4. Serial injection of 40 consecutive analyses of polyamine standards (1 mM) and their deuterated internal standards when using MSI-CE-MS as a high throughput platform for metabolomics. Abbreviations: DiAc, diacetyl; Spm, spermine; Ac, acetyl; and Spd, spermidine (Reproduced from Igarashi et al., 2021).

polyamine concentrations measured by MSI-CE-MS as compared to conventional single injection CE-MS and LC-MS/MS methods (Igarashi et al., 2021). However, reversed-phase LC-MS remains the central platform for lipidomic studies as water-insoluble lipids are not measured by CE-MS protocols using aqueous background electrolyte systems. However, recent advances in developing multisegment injection-nonaqueous capillary electrophoresis-mass spectrometry (MSI-NACE-MS) offer a rapid yet multiplexed separation platform for the nontargeted analysis of diverse classes of ionic lipids differing in polarity in serum extracts, such as phosphatidylcholines, phosphatidic acids, phosphatidylinositols and nonesterified fatty acids (Azab et al., 2019; Ly et al., 2022). MSI-NACE-MS is anticipated to greatly increase overall metabolome coverage beyond polar metabolites given the large number of lipids present in blood specimens that requires better reporting

standards and data harmonization (Bowden et al., 2017).

4　Conclusions and Future Perspectives

Over the past twenty year, Dr. Masaru Tomita has established an exceptional metabolomics facility in rural and northern Japan featuring the largest number of operating CE-MS instruments worldwide. The development of robust and standardized CE-MS protocols together with software packages for data pre-processing and visualization have been critical to influential breakthroughs in translational science when using nontargeted metabolite profiling for biomarker discovery. These include CE-MS metabolomic applications in integrative systems biology to evaluate perturbations and multiple gene knockout mutations on *E. coli* (Ishii et al., 2007; Nakahigashi et al., 2009), metabolite indicators associated with sensory evaluation scores of Japanese sake or dry cured ham (Sugimoto et al., 2010c; Sugimoto et al., 2020), and diagnostic biomarkers for oral and colorectal cancer screening (Ishikawa et al., 2016; Kuwabara et al., 2022). His sustained efforts have also supported local companies and community residents living in Tsuruoka city while fostering a diverse array of engaging collaborations from across Japan and internationally. Moreover, several successful bio-venture spin-off companies have also been founded based on the fundamental metabolomics research program at IAB, including Human Metabolome Technologies Inc, Saliva Tech. Co., and Spiber Inc. The impact of large-scale metabolomics studies to assess environmental exposures that modulate chronic disease risk is still underway, which may reveal preventative dietary and lifestyle patterns to promote human health, well-being and longevity. Future technology development in CE-MS promises to further enhance concentration sensitivity, sample throughput, data quality, and metabolome coverage to encompass a wide range of ionic lipids in complex biological samples.

Acknowledgements

P.B.M. acknowledges funding from the Natural Sciences and Engineering Research Council of Canada, Cystic Fibrosis Canada, the Canada Foundation for Innovation and Genome Canada.

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〔受付日　2022. 8. 13〕