A Robust and High Throughput Method for Anionic Metabolite Profiling: Preventing Polyimide Aminolysis and Capillary Breakages under Alkaline Conditions in CE-MS

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Capillary electrophoresis-mass spectrometry (CE-MS) represents a high efficiency microscale separation platform for untargeted profiling of polar/ionic metabolites that is ideal for volume-restricted biological specimens with minimal sample workup. Despite these advantages, the long-term stability of CE-MS remains a major obstacle hampering its widespread application in metabolomics notably for routine analysis of anionic metabolites under negative ion mode conditions. Herein, we report for the first time that commonly used ammonia containing buffers compatible with electrospray ionization (ESI)-MS can compromise the integrity of fused-silica capillaries via aminolysis of their outer polyimide coating. Unlike organic solvent swelling effects, this chemical process occurs under aqueous conditions that is dependent on ammonia concentration, buffer pH and exposure time resulting in a higher incidence of capillary fractures and current errors during extended operation. Prevention of polyimide aminolysis is achieved by using weakly alkaline ammonia containing buffers (pH < 9) in order to preserve the tensile strength of the polyimide coated fused-silica capillary. Alternatively, less nucleophilic primary/secondary amines can be used as electrolytes without polyimide degradation, whereas chemically resistant polytetrafluoroethylene coating materials offer higher pH tolerance in ammonia. In this work, multi-segment injection (MSI)-CE-MS was used as multiplexed separation platform for high throughput profiling of anionic metabolites when using optimized buffer conditions to prevent polyimide degradation. A diverse range of acidic metabolites in human urine were reliably measured by MSI-CE-MS via serial injection of seven urine samples within a single run, including organic acids, food-specific markers, microbial-derived compounds and over-the-counter drugs as their sulfate and glucuronide conjugates. This approach offers excellent throughput (< 5 min/sample) and acceptable intermediate precision (average *CV* ≈ 16%) with high separation efficiency as reflected analysis of 30 anionic metabolites following 238 repeated sample injections of human urine over 3 days while using a single non-isotope internal standard for data normalization. Careful optimization and rigorous validation of CE-MS protocols are crucial for developing a rapid, low cost and robust screening platform for metabolomics that is amenable to large-scale clinical and epidemiological studies.

INTRODUCTION

Although developed primarily as synthetic tubing materials for the fiber optic telecommunications industry,[1] the advent of open tubular fused-silica capillary columns has revolutionized separation science with the introduction of high efficiency microscale analytical techniques, including gas chromatography (GC), nano-liquid chromatography (nano-LC) and capillary electrophoresis (CE).[2] Polyimide is the outer polymer coating material of choice in the manufacture of rugged fused-silica capillaries due to its excellent thermal stability and chemical resistance that imparts flexibility with abrasive protection to prevent breakage. Despite these characteristics, polyimide coated fused-silica capillaries are susceptible to random brittleness problems during the capillary drawing process, including variations in inner surface activity and capillary dimensions that can impact separation performance.[3] Polyimide coated fused-silica capillaries are also prone to swelling after prolonged contact with certain organic solvents (*e.g.*, acetonitrile) that leads to clogging events and capillary breakage in non-aqueous CE and capillary electrochromatography (CEC).[4] Extensive heat curing prior to use has been reported to provide greater tolerance in organic solvents that maintain separation efficiency with longer capillary lifetimes.[5] Alternatively, a section of the polyimide coating at the capillary inlet/outlet in contact with organic solvent can be stripped chemically with concentrated nitric acid or charred by heating,[6] however this step can compromise mechanical stability. The latter procedure is often adopted in CE-MS methods with a coaxial sheath liquid interface in order to prevent swelling effects since a make-up flow of an electrolyte solution containing organic solvent is required for stable electrospray generation.[7] Similarly, more sensitive sheathless porous tip interface designs in CE-MS rely on an uncoated and thin walled/etched fused-silica tubing to ground the electrical circuit – a fragile section of the capillary requiring careful handling to prevent fractures or breakage.[8] As a result, the robustness of CE-MS technology remains an on-going challenge that has limited its application by a wider community of researchers involved in large-scale metabolomic profiling of complex biological fluids,[9] despite recent advances at improving concentration sensitivity, migration time reproducibility and sample throughput.[10]

Several studies have demonstrated that CE-MS offers complimentary selectivity in metabolomics in comparison to both reversed-phase LC and hydrophilic interaction chromatography (HILIC) notably for strongly ionic and poorly retained metabolites in volume-

restricted or mass-limited biological samples, including bio-banked specimens.[11-14] Overall, there is growing consensus that excellent performance is achieved for untargeted profiling of metabolites and peptides by CE-MS under strongly acidic conditions using acetic acid or formic acid as a background electrolyte (BGE) with positive ion mode detection.[15-17] For instance, a recent inter-lab validation study for tryptic peptide mapping by CE-MS using a standardized operating protocol demonstrated good method robustness across 13 independent laboratories from academia and industry when using different ion source interfaces and mass analyzers.[18] Indeed, CE-MS metabolomic studies to date have primarily focused on comprehensive profiling of cationic metabolites[19-26] given the lower sensitivity and poor stability long associated with anionic metabolites under negative ion mode detection.[27] Büscher *et al.*[28] reported a cross-platform study for quantitative metabolomic analyses of yeast extracts that highlighted CE-MS suffers from poor reliability in comparison to LC-MS and GC-MS, which was attributed to the protocol used for anionic metabolite profiling. This problem was later linked to incidental corrosion of the stainless steel spray needle causing clogging of the capillary outlet with frequent current drops while operating CE-MS under reversed polarity with a cationic polymer-coated capillary[29] several years after publication of their original protocol[30] and subsequent applications.[31, 32] As a result, there have been sparse reports on the use of uncoated capillaries using simple alkaline buffers for anionic metabolite profiling using CE-MS under normal polarity configuration.[33] Kok *et al.*[34, 35] compared different background electrolyte (BGE) and sheath liquid additives for anionic metabolite profiling and concluded that triethylamine (pH 11.7) provided better sensitivity and greater metabolome coverage than conventional ammonia containing buffers. However, persistent "memory effects" when using the same instrument under positive ion detection occurs since TEA strongly adsorbs onto surfaces that contributes to ion suppression due to its high surface activity and gas phase proton affinity.[36] Recently, Gulersonmez *et al.*[37] developed a sheathless CE-MS method for reliable analysis of anionic metabolites (*e.g.*, sugar phosphates) from glioblastoma cell extracts when using acetic acid as the BGE (pH 2.2) with negative ion mode detection. However, this approach is viable for separating only strongly acidic metabolites (pK_a < 3.2) while being limited to small sets of biological samples due to the finite lifespan of the porous-etched capillary tip. Thus, there is an urgent need for a simple, cost effective yet robust CE-MS platform amenable to large-scale metabolomic investigations involving complex biological samples, such as human urine that is composed of a

diverse range of acidic/ionic compounds reflecting diet, chemical exposures, gut microflora and host metabolism.[38]

Herein we demonstrate for the first time that alkaline aqueous ammonia solutions widely used in ESI-MS lead to chemical degradation of the outer polyimide coating, which compromises the tensile strength of fused-silica capillaries during extended operation in CE-MS. Aminolysis of polyimide results in an increased incidence of capillary breakages that are prevented when using mildly basic ammonia solutions (pH < 9) at low ionic strength, ammonia-free or less nucleophilic primary or secondary amine buffers, as well as chemically resistant polytetrafluroethylene coated capillaries. A multiplexed separation platform based on multisegment injection (MSI)-CE-MS[24] was rigorously validated for high throughput anionic metabolite profiling when analyzing 238 repeated sample injections of pooled 24 h urine over 3 days under optimized buffer conditions that avoid polyimide dissolution. Accurate prediction of the electromigration behaviour of diverse classes of acidic metabolites in human urine is also demonstrated to assist in the identification of urinary metabolites in cases when authentic standards or MS/MS spectra are unavailable. This work sheds new light into longstanding concerns regarding the robustness of CE-MS technology highlighting the need for careful attention to buffer compatibility in method development.

EXPERIMENTAL

Chemicals and Reagents. All chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) unless otherwise stated. Deuterium oxide (D, 99.9%) was purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA). Stock solutions for all internals standards and metabolite standards, as well as background electrolyte (BGE) and sheath liquid solutions were prepared in deionized water from a Barnstead EASYpure® II LF system (Dubuque, IA, USA). HPLC-grade methanol and acetonitrile were used to prepare sheath liquid and BGE solutions, respectively. All stock solutions for chemical standards were stored at 4°C.

CE-MS Instrumentation. All flexible open tubular fused-silica capillaries with standard polyimide (TSP) or deep UV transparent polytetrafluoronated (TSU) outer coatings were purchased from Polymicro Technologies Inc. (Phoenix, AZ, USA) with inner diameters of 50 μm

and outer diameters of 363 μm. CE-MS experiments were performed on an Agilent G7100A CE instrument (Agilent Technologies Inc., Mississauga, ON, Canada) equipped with a coaxial sheath liquid Jetstream electrospray ion source with heated nitrogen gas, which were coupled to an Agilent 6230 TOF-MS system. Separations were performed using a 110 cm polyimide coated fused-silica capillary using an applied voltage of 30 kV under normal polarity configuration at 25°C. In order to reduce total analysis times, a hydrodynamic pressure gradient was also applied during voltage application at a rate of 1 mbar min^{-1} or 0.1 kPa min^{-1} from 10 to 20 min, which was then increased to 3 mbar min^{-1} or 0.3 kPa min^{-1} from 20 min until the end of the separation. The background electrolyte (BGE) consisted of 50 mM ammonium bicarbonate, pH 8.50 that was adjusted with 1.5 M ammonium hydroxide unless otherwise stated, whereas the sheath liquid was composed of 50:50 MeOH:H_2O. New capillaries were conditioned by flushing at high pressure (900 mbar or 90 kPa) each morning for 15 min each in the order of MeOH, 0.1 M NaOH, deionized H_2O, 1 M formic acid, and deionized H_2O followed by BGE. In between runs, the capillary was flushed using deionized H_2O for 3 min followed by equilibration with BGE for 5 min. A 7-sample segment serial injection format was used for MSI-CE-MS as a multiplexed separation platform[24] to enhance sample throughput for anionic metabolite profiling under negative ion mode detection. This was performed by a programmed hydrodynamic injection sequence at 100 mbar or 10 kPa comprising seven alternating human urine and BGE plugs that is equivalent to filling about one third of total capillary length, namely (1) 5 s sample, 40 s BGE spacer, (2) 5 s sample, 40 s BGE spacer, (3) 5 s sample, 40 s BGE spacer; (4) 5 s sample, 40 s BGE spacer; (6) 5 s sample, 40 s BGE spacer; (7) 5 s sample, 5 s BGE spacer. Between runs, the capillary was flushed at high pressure (900 mbar or 90 kPa) with deionized water for 5 min and then BGE for 10 min. The TOF-MS was operated under negative-ion mode conditions that spanned a mass range of *m/z* 50-1700, with an acquisition rate of 500 ms/spectrum. The ESI conditions were Vcap = 2000 V, nozzle voltage = 2000 V, nebulizer gas =10 psi, sheath gas =3.5 L/min at 195 °C, drying gas 8 L/min at 300 °C. Also, the MS voltage settings were fragmentor = 120 V, skimmer = 65V and Oct1 RF = 750 V.

Tensile Strength of Fused-silica Capillaries Exposed to Alkaline Buffer Solutions. Passive exposure studies on 2 x 5 cm length segments of polyimide-coated or polytetrafluoroethylene coated fused-silica capillaries (50 μm ID; 363 μm□OD) were performed in triplicate in three

separate 15 mL plastic centrifuge tubes. All six solution-exposed capillaries were then tested for changes in tensile strength by a single volunteer who manually bent the capillary to 90^0 every day for the first week and then every third day in subsequent weeks for a period of 26 days. The day upon which a capillary fractured with bending force following buffer exposure was noted and the broken edges were inspected using a Nikon ECLIPSE LV100N POL microscope equipped with objective lens and Q IMAGING RETIGA 2000R camera (Nikon Canada, Mississauga, ON, Canada). Various aqueous alkaline buffer solutions were examined in this study whose composition varied in terms of electrolyte type (*i.e.*, with/without ammonia), ionic strength, and buffer pH, which were left at room temperature. 50 mM borate and 50 mM ethylamine buffers at pH 10.0 (pH adjusted with 1M formic acid) were used as controls relative to 50 mM ammonium bicarbonate buffer at pH 8.5, 9.3 and 10 (pH adjusted with 1.5 M ammonium hydroxide), as well as 50 mM or 100 mM ammonium bicarbonate at pH 10 (pH adjusted with 1 M LiOH). In addition to evaluating the chemical stability of conventional polyimide coated capillary tubes to alkaline ammonia-based solutions, polytetrafluoroethylene coated capillary segments were also compared as a control. To assess accelerated degradation by aminolysis during high pressure flushing of solution in CE, a 110 cm polyimide fused-silica capillary was continuously flushed for 24 h with 50 mM ammonium bicarbonate buffer at pH 8.5 or 10.0 using a pressure of 900 mbar or 90 kPa. The inlet and outlet section of each capillary end was then imaged under the light microscope to confirm changes in the morphology of polyimide coating as a function of buffer pH (pH > 9) that was also associated with reduced tensile strength, increased fragility, shorter lifespans and polyimide dissolution following prolonged exposure and/or flushing at high pressure.

Characterization of Ammonia-induced Degradation Products in Solution. Aliquots of the brown-coloured solutions were taken from completely solubilized polyimide from bare fused-silica capillaries following prolonged exposure (> 120 days) in 100 mM ammonium bicarbonate, pH 10. Blank-corrected UV-vis absorbance spectra were acquired from a five-fold diluted sample of polyimide solution in 50 mM borate, pH 9.0 using a Carey 50 spectrophotometer (Varian Inc., Palo Alto, CA, USA) at room temperature with high resolution scanning from 200 nm to 800 nm. ^{1}H-NMR studies were performed using a Bruker AV600 MHz spectrometer following solvent evaporation of a 0.25 mL samples using a Vacufuge Plus system (Eppendorf

Inc. Mississauga, ON, Canada), reconstituted to 0.5 mL in deuterium oxide (D_2O) and transferred into a 5 mm id NMR tube. Chemical shifts and integrated proton signals for three distinct chemical environments were measured in ^1H NMR spectra. Chemical degradation by-products of the solubilized polyimide coating were identified by MSI-CE-MS when using a dilution trend filter in MSI-CE-MS[26] together with Molecular Feature Extractor in Mass Hunter software (Agilent Technologies Inc.). Samples were analyzed using 50 mM ammonium bicarbonate, pH 8.5 as BGE under negative ion mode (anionic metabolites), as well as 1M formic acid, pH 1.8 under positive ion mode (cationic metabolites), which confirmed the detection of six unknown species in solution following polyimide degradation (two anionic and four cationic species) defined by their *m/z:*RMT and most likely elemental formula.

Sample Pretreatment of Human Urine Samples. All human urine samples analyzed were pooled 24 h urine specimens derived from the Prospective Urban Rural Epidemiological Study (PURE) as described in our previous study for urinary iodine status determination.[39] Urine samples were stored frozen at -80°C prior to their thawing at room temperature. Prior to analysis, about 50 μL of urine was centrifuged at 1,500 *g* for 5 min to precipitate particulate matter, then the supernatant was diluted five-fold with deionized water containing internal standard (50 μM NMS). Technical replicates of the pooled specimens (n = 20) were prepared in advance and kept frozen at –80 °C. Fresh aliquots were thawed three times daily and exchanged every five analysis runs in MSI-CE-MS.

Optimization of Background Electrolyte for Anionic Metabolite Profiling. In order to avoid aminolysis of polyimide coated fused-silica capillaries to enable robust profiling of acidic metabolites in urine by MSI-CE-MS with negative ion mode detection, volatile ammonium containing buffers were limited to a pH 8.5 unlike primary or secondary amine buffers (*e.g.,* ethylamine). All BGEs tested were compared in terms of their impact on peak resolution and separation efficiency for representative urinary metabolites, as well as overall detector response based on measured signal-to-noise ratio (*S/N*) for ions. Five equimolar BGE systems (50 mM) were evaluated for MSI-CE-MS using a 7 serial plug injection format for diluted (5-fold in de-ionized water) pooled human urine, including ammonium bicarbonate (pH 8.5), ammonium acetate (pH 8.5), ethylamine (pH 10), diethylamine (11.8) and pyrrolidine (pH 12). MSI-CE-MS

analyses were performed over two day using the same capillary and at least four sets of data were collected for each buffer system.

Intermediate Precision for Anionic Metabolite Profiling of Urine. Daily mass tuning, cleaning of CE electrode (at inlet) and capillary conditioning steps were conducted by a single individual each morning using a standardized operating protocol to minimize sample carryover and systematic bias. The ion source was also rinsed with 50:50 isopropyl alcohol:H_2O and dried for 30 min prior to starting. In addition, a new polyimide coated fused-silica capillary, sheath liquid solution and BGE for CE-MS were prepared each day over three days in order to introduce random variation to the analyses. A standard metabolite mixture containing 20 organic acids with internal standard (50 μM NMS) was then analyzed each morning to check the stability of the CE current, as well as migration times, resolution and ion responses for representative urinary metabolites (*e.g.*, uric acid, indoxyl sulfate etc.). MSI-CE-MS runs that were observed to have significantly low currents or irreversible current drops were excluded from data analysis due to instrumental bias. This problem was found to occasionally arise (3 out of 45 runs) as a result of improper alignment of capillary inlet with vial during sample injection leading to incidental injection of an air plug. Each morning following conditioning of a new fused-silica capillary, a standard mixture of anionic metabolites was analyzed followed by two consecutive equilibration runs of a five-fold diluted urine were performed by MSI-CE-MS using a 7-sample plug format for stabilization of the electroosmotic flow (EOF) with urine matrix prior to completion of about 15 runs or 105 sample injections comprising three technical replicates of urine (prepared every 5 runs) per day. A fresh BGE vial was introduced every four MSI-CE-MS runs to avoid buffer depletion and sample carry-over, whereas a fresh aliquot of diluted pooled urine sample was replaced every 8 runs to prevent evaporation losses. Between runs, the capillary was flushed at high pressure (900 mbar or 90 kPa) with deionized water for 5 min and then BGE for 10 min. A total of 45 runs were performed by MSI-CE-MS over three days, which included a standard mixture and urine samples for equilibration each morning, as well as spiked samples required for urinary metabolite identification. After rejecting runs that exhibited current drops, 34 runs acquired over 3 days equivalent to a total of 238 individual injections (34 runs x 7 injections per run = 238 total injections) of the same pooled 24 h urine sample were processed for inter-day reproducibility studies. The migration times and integrated peak areas for 30 representative

acidic metabolites in urine were processed using Mass Hunter Qualitative Analysis software v B.06.00 SP (Agilent Technologies Inc.) and then normalized relative to NMS as internal standard to correct for differences in EOF and in-capillary injection volume between samples, respectively.[24] The intermediate precision of MSI-CE-MS was evaluated in terms of relative migration times (RMTs) and relative peak areas (RPA) for confirmed (*i.e.*, identified with authentic standards) and putative anionic metabolites (*i.e.*, denoted by unique *m/z*:RMT and likely molecular formula with corresponding mass error) in human urine. All data processing and statistical analysis was performed using Igor Pro 5.0 (Wavemetrics Inc., Lake Oswego, OR, USA) and Excel (Microsoft Inc., Redmond, WA, USA).

Modeling of Ion Migration Behavior To Support Metabolite Identification. A chemically diverse set of 30 acidic metabolites consistently detected in pooled 24 h human urine samples using MSI-CE-MS (*e.g.*, mono- and divalent organic acids, zwitter-ions, glucuronide and sulfate conjugates etc.) were selected as a training set to accurately predict the relative migration time (RMT) of an ion (relative to NMS) based its absolute electrophoretic mobility (μ_o) as described by the Hubbard-Onsager equation[40] that includes the impact of hydrodynamic and dielectric friction for describing ion migration behavior in aqueous solutions at infinite dilution:

$$\mu_0 = \frac{az_0}{V^c + (\frac{bz_0^2}{V})} \qquad (1)$$

Thus, two fundamental parameters are needed for determination of μ_o as derived from the chemical structure of a putative ion, namely its effective charge (z_o at pH 8.5) and molecular volume (V), whereas coefficient terms used in *eq (1)* are a = 0.00132, b = -18.8 and c = 0.335 as previously described.[41] In this work, z_o was determined using pK_a values listed in the Human Metabolome Database (http://www.hmdb.ca) based on the effective charge state of an acidic urinary metabolite at pH 8.5 for CE separation using the Henderson-Hasselbach equation. Also, V was determined *in silico* using Chem3D Pro software (Cambridge Soft, Cambridge, MA, USA), where each chemical structure was energy-minimized using an iterative molecular mechanics 2 (MM2) algorithm to determine a stable molecular conformation prior to computation of its Connolly solvent-excluded molecular volume. A correlation plot between experimentally measured RMTs for 30 urinary metabolites and predicted μ_o, demonstrated good

linearity (R^2 = 0.974) and accuracy (average bias = +0.3% with range from +7.6% to -9.1%). This approach was used to confirm the assignment of putatively identified urinary metabolites based on its accurate mass and most likely molecular formula in cases when authentic standards were not available or precursor ion signals were too low for acquisition of MS/MS spectra.

RESULTS AND DISCUSSION

Our previous study[42] demonstrated excellent selectivity for resolving weakly acidic native estrogens and their intact sulfate and glucuronide conjugates in urine, including positional isomers when using CE-MS. However, sporadic capillary breakage and current instability events with prolonged usage of aqueous buffers containing ammonia prompted us to further investigate the underlying cause of this problem, which was unrelated to organic solvent-induced swelling or anodic corrosion of sprayer. **Figure 1(a)** depicts representative images of short sections of polyimide coated fused-silica capillaries following long-term exposure (70 days) to aqueous alkaline solutions that vary in terms of electrolyte type, buffer pH and ionic strength. It was apparent that exposure to 50 mM ammonium bicarbonate, pH 10 (or ammonium acetate) resulted in softening/warping of the outer polyimide coating with gradual dissolution of the polymer into solution. In fact, the first evidence of distortion of the polyimide coating was observed within 12 days first noticeable at the cut ends of the capillary, whereas complete dissolution/stripping of polyimide occurred within 2 days when exposed to 1 M ammonium hydroxide. This effect was not observed following exposure studies performed in equimolar "ammonia-free" aqueous alkaline buffers (50 mM borate, pH 10) or primary/secondary amine buffer electrolytes, such as ethylamine (pH 10) as shown in **Figure 1(a)**. Similarly, no changes in polyimide coating morphology or polymer dissolution were apparent after prolonged exposure to 50 mM ammonium bicarbonate, pH 8.5. Since passive contact of polyimide coated fused-silica capillaries do not reflect typical use in CE, **Figure 1(a)** also depicts two microscopic images near the capillary inlets acquired following 24 h of continuous flushing at high pressure (900 mbar or 90 kPa) using 50 mM ammonium bicarbonate. This study clearly highlights that softening and elongation of the outer polyimide coating beyond the tip of the capillary is evident at pH 10, which did not occur when flushed with the same buffer at pH 8.5. This effect is similar to previous reports of acetonitrile-induced polyimide swelling of fused-silica capillaries in CEC and

non-aqueous CE, where the swollen polyimide coating extending beyond the capillary inlet has been attributed to several deleterious effects, such as sample injection variability or sudden current drops during voltage application.[4-6] However, unlike organic solvent-induced swelling effects, exposure to aqueous alkaline ammonia solutions leads to chemical degradation and dissolution of the outer polyimide coating rendering the fused-silica capillary extremely fragile.

Figure 1(b) also compares changes in tensile strength and resistance to fracture of fused-silica capillaries following application of a repeated bending force (90° angle) performed manually over a 26 day period during solution exposure. This study clearly demonstrates that early stages of polyimide degradation coincided with a higher incidence of capillary breakages and a dramatically shorter average lifespan of only 2 days when placed in 100 mM ammonium bicarbonate, pH 10. In contrast, immersion into 50 mM ammonium bicarbonate, pH 8.5 did not undergo any capillary breakage events during the entire 26 day exposure period. As expected, an intermediate pH of ammonium bicarbonate (pH 9.3) resulted in a reduced average lifespan of about 17 days, whereas pH adjustment of 50 mM ammonium bicarbonate, pH 10 with lithium hydroxide increased average lifespan by about 8 days relative to the use of ammonium hydroxide since the latter pH adjustment additive increases the effective concentration of ammonia in solution. These observations are consistent with the much lower fraction of reactive ammonia existing at equilibrium in solution at pH 8.5 relative to pH 10 (pK_a = 9.25). Overall, aqueous buffer solutions containing elevated ammonia concentrations at higher pH conditions accelerated polyimide degradation while increasing the susceptibility of the fused-silica capillary tubing to fracture, which was prevented by using more chemically resistant polytetrafluoroethylene coated capillaries, “ammonia-free” alkaline buffers (*e.g.*, borate) or less nucleophilic primary/secondary amines (*e.g.*, ethylamine, diethylamine, pyrrolidine) as BGE. To the best of our knowledge, only one previous technical report by NASA[43] has examined the chemical stability of polyimide when exposed to caustic solutions (pH 11-14), which indicated only modest swelling effects depending on chemical structure of the specific polyimide, as well as polymer morphology, and curing status. Aqueous degradation of Kapton-based polyimide films under strongly acidic conditions (pH < 2) have also been attributed to hydrolysis of susceptible aromatic imide and amide functional groups that ultimately reduce the tensile strength of the polymer with a corresponding decrease in elongation to failure.[44] However, our studies clearly demonstrate that polyimide

coated fused-silica capillaries used in CE and nano-LC are stable in "ammonia-free" alkaline buffer solutions, but are susceptible to aminolysis at pH > 9 despite the widespread use of ammonia containing buffers as volatile electrolytes in ESI-MS. Although the exact composition of the commercial polymer coating is not known, aromatic polyimides containing a heterocylic imide linkage in their repeat unit are typically used in manufacture due to their high thermal stability.[45] **Figure S1 in the Supplemental Information** section provides evidence that irreversible dissolution of the fused-silica capillary outer coating results in formation of a brown-orange solution that is consistent with an aromatic polyimide precursor as indicated by ^{1}H-NMR and UV-vis absorbance. Also, MSI-CE-MS using a dilution trend filter with molecular feature extraction[24] revealed the presence of two acidic (anionic) and four basic (cationic) ions as by-products of polyimide degradation as defined by their characteristic *m/z*:RMT under negative and positive ion mode detection, respectively. A PubChem search for these unknown synthetic compounds using their top three ranked molecular formulae revealed no known chemical structures on the database in the case for anions, and a large number of putative candidates and their isomers (> 120) for cations. Thus, a total of six highly unsaturated, aromatic, nitrogen-rich, and water-soluble ions were identified as novel polyimide aminolysis by-products in solution. The chemical stability of polyimide coated open tubular capillaries examined in this work was derived from a single manufacturer, thus considerable differences are expected in products from other companies using different proprietary polyimide coatings and heat curing processes.

A systematic study was next performed by comparing the performance of five different alkaline BGEs that are compatible for anionic metabolite profiling of pooled 24 h urine samples when using MSI-CE-MS with conventional polyimide coated capillaries, including equimolar concentrations (50 mM) of ammonium bicarbonate (pH 8.5), ammonium acetate (pH 8.5), ethylamine (pH 10.0), diethylamine (pH 11.0) and pyrrolidine (pH 11.3). Both caustic solutions containing diethylamine and pyrrolidine produced stable yet low currents (< 15 μA) with a fast EOF resulting in a narrow separation time window and lower peak capacity for separation. Overall, ethylamine was found to generate slightly better sensitivity for detection of anionic urinary metabolites under negative ion mode relative to ammonium acetate as BGE.[34, 35] However, ammonium bicarbonate provided far better performance in terms of improved ion signals, higher separation efficiency and shorter migration times for a diverse array of anionic

metabolites in human urine without compromising the integrity of the polyimide outer coating as depicted in **Figure S2 in the Supplemental Information** section. Indeed, enhanced sensitivity was acquired using ammonium bicarbonate as BGE as reflected by a 50% increase in total molecular features detected from diluted urine samples relative to ammonium acetate or ethylamine since electrolyte type can differentially impact solute ionization efficiency.[27] In our study, no electrolyte was added to the sheath liquid solution (50:50 MeOH:H_2O), which ultimately minimized the impact of ion suppression by bicarbonate in the BGE due to the much higher flow rate of sheath liquid used for spray formation. **Figure 2(a)** depicts a schematic of the serial injection configuration used in MSI-CE-MS that relies on repeated hydrodynamic injection of alternating plugs of urine (five-fold diluted in deionized water) and BGE prior to zonal separation of acidic urinary metabolites from each sample plug under steady-state electrophoretic and ionization conditions. Also, **Figure 2(b)** highlights an overlay of current traces from 45 runs performed over 3 days when using MSI-CE-MS, where each run is comprised of a multiplexed analysis of 7 (or more) samples as a way to improve sample throughput without complicated column switching programs, which increases overall productivity of MS-based metabolomics without added infrastructure costs.[24] The isocratic elution conditions of CE together with the homogenous ionization conditions provided by the sheath liquid ensures that the same metabolite entering the ion source offset in time from different samples generates a series of resolved peaks as shown in **Figure S3 of the Supplemental Information** section. Absolute quantification is also feasible given the high efficiency separation of urine matrix interferences when using a single non-isotope internal standard for correction of between-sample variations in injection volume.[24-26] Monitoring current traces in CE-MS provides an effective way to evaluate system stability and detect systematic error as evident by 3 runs that had a significantly lower current and/or sudden current drops, which originated from misalignment of the inlet capillary during sample loading. Overall, the CE separation current was stable and reproducible after the migration of neutral compounds with the electroosmotic flow (EOF, $\approx$ 11 min) in the majority of runs with an overall CV = 2.6% (n = 42) over 3 days of operation despite installing/conditioning a new capillary, as well as preparing a fresh BGE/sheath liquid solution and three technical replicates of diluted urine samples each day. Each morning started with a wipe down of platinum electrodes at inlet and ion source to reduce sample carry-over followed by analysis of a standard mixture of anionic metabolites and two equilibrating runs of pooled urine samples. As a result,

34 runs or 238 repeated sample injections of pooled 24 h human urine were used to evaluate the long-term performance of MSI-CE-MS for routine anionic metabolite profiling in complex biological samples.

Figure 2(c) depicts a representative total ion electropherogram (TIE) of the multiplexed analysis of 7 diluted human urine samples in series by MSI-CE-MS, which highlights that effective desalting of inorganic electrolytes (*e.g.*, Na^+ or salt front) and neutral compounds that co-migrate with the EOF (*e.g.*, creatinine) precedes the migration of major acidic metabolites in human urine, such as phenylacetylglutamine and uric acid. **Figure 2(d)** illustrates extracted ion electropherograms (EIE) for three distinct classes of strongly ionic and weakly acidic urinary metabolites that vary in their signal intensity when using MSI-CE-MS, including indoxyl sulfate, *D*-quinic acid (resolved from the later migrating tricarboxylic acid isobar, citric acid), and ibuprofen 3-glucuronide. For instance, indoxyl sulfate is a uremic toxin associated with vascular dysfunction,[46] whereas *D*-quinic acid is a dietary phytochemical with poorly understood biological functions that is aromatized to hippuric acid by action of gut microflora.[47] High dose intake of the analgesic ibuprofen (excreted in urine as hydroxyibuprofen and its intact glucuronide conjugate) is associated with increased risk of myocardial infarction for subjects with pre-existing cardiovascular disease.[48] Effective sample throughput is increased significantly since seven samples are analyzed within the same run without loss in resolution or information content that maximizes the peak capacity of the separation. Moreover, quality control (QC) samples can be introduced within each run to assess or correct for systematic error over a large batch of runs, whereas signal pattern recognition of time-resolved signals enables unambiguous molecular feature selection for untargeted metabolite profiling that reduces false discoveries.[24-26] Each urinary metabolite is defined by its characteristic mass-to-charge ratio (*i.e.*, accurate mass) and relative migration time as a paired variable (*m/z*:RMT) together with its likely molecular formula as summarized in **Table 1** for 30 different ionic metabolites ranging from small organic acids (*e.g.*, lactic acid), microflora-derived metabolites (*e.g.*, *o*-cresol sulfate), endogenous steroid (*e.g.*, tetrahydroaldosterone 3-glucuronide) or exogenous over-the-counter drug conjugates (*e.g.*, acetaminophen sulfate). In fact, **Figure 2(d)** highlights that later migrating metabolites (*e.g.*, indoxyl sulfate) from samples introduced at the end of the serial injection sequence in MSI-CE-MS elute with sharper peaks despite longer times for diffusion likely as a

result of sample self-stacking (*i.e.*, transient isotachophoresis) when analyzing highly saline urine samples.[39] This effect was not attributed to ion suppression or enhancement effects for acidic metabolites migrating after the salt front and EOF as confirmed by monitoring changes in the ion signal of internal mass calibrants added to the sheath liquid during the time course of the separation as shown in **Figure S4 in the Supplemental Information**.

Figure 2(e) shows control charts based on integrated peak area ratios normalized to a single non-isotope internal standard (50 μM NMS) following 238 repeated sample injections (or 34 runs) by MSI-CE-MS over 3 days of analysis while applying a standardized operating protocol. Overall, controls charts indicate good intermediate precision without evidence of systematic error as reflected by coefficient of variance (*CV*) ranging from 8.8% to 21.8% depending on ion abundance. **Table 1** shows that the overall *CV* for 30 different urinary metabolites was about 16% in terms of relative peak area ratios, whereas it was about 0.9% for RMTs. In this case, the IS is primarily used to correct for variations in injection volume between samples in MSI-CE-MS, whereas improved precision is expected if co-migrating deuterated ISs are included for each metabolite in order to correct for changes in spray stability during separation.[49] In most cases, metabolite identification was confirmed after spiking authentic standards in urine when available, whereas some urinary metabolites were tentatively assigned based on their accurate mass and most likely molecular formula with a mass error < 5 ppm in most cases (with exception of low abundance metabolites), as well as characteristic electromigration behaviour in aqueous solution (*i.e.*, predicted RMTs). **Figure S5 and Table S1 of the Supplemental Information section** demonstrate that the absolute mobility of anionic metabolites can be well described by two intrinsic physiochemical properties of an ion when using the Hubbard-Onsager equation,[44] namely the effective charge at pH 8.5 (z_{eff}) as derived from its pK_a and the solvent-excluded molecular volume (V). Overall, a good correlation (R^2 = 0.974) was found between the average RMT of 30 urinary metabolites and their predicted absolute mobility as derived from their chemical structure. Thus, CE offers a complimentary approach to MS for prioritizing putative isobaric candidates for biomarker discovery in metabolomics in cases when mass spectral databases are incomplete while also confirming likely isomeric compounds not readily distinguished by MS/MS.[41]

CONCLUSION

In summary, this is the first report to describe the deleterious effects of polyimide degradation when using flexible open tubular fused silica capillaries after prolonged exposure to aqueous alkaline ammonia buffers. Irreversible aminolysis of the outer polyimide coating results in polymer dissolution while severely decreasing the tensile strength of the capillary during extended operation with poor long-term stability. Our work demonstrates that MSI-CE-MS offers a robust and high throughput screening platform for characterization of the ionic metabolome,[50] such as a diverse range of acidic metabolites in human urine when operating under optimum conditions to prevent polyimide aminolysis and capillary breakages. Putative identification of metabolites in the absence of authentic standards is facilitated by accurate modeling of the electromigration behaviour *in silico* in conjunction with high resolution, accurate MS notably when MS/MS spectra cannot be acquired, such as the case for low abundance or weakly ionizable precursor ions. Future work will explore the use of alternative polymer materials that better tolerate strongly alkaline ammonia solutions and organic solvents while overcoming the limited thermal stability of polytetrafluoroethylene coatings. Inter-laboratory validation studies of MSI-CE-MS technology under standardized operating conditions are in progress to facilitate method translation to new users and different biological samples, including round-robin studies to assess the analytical performance relative to other techniques widely used in metabolomics, including ^{1}H-NMR, GC-MS and LC-MS. MSI-CE-MS offers a rapid, low cost yet reliable method for untargeted profiling of polar/ionic metabolites in complex biological samples that maximizes the productivity of MS infrastructure without compromising separation performance.

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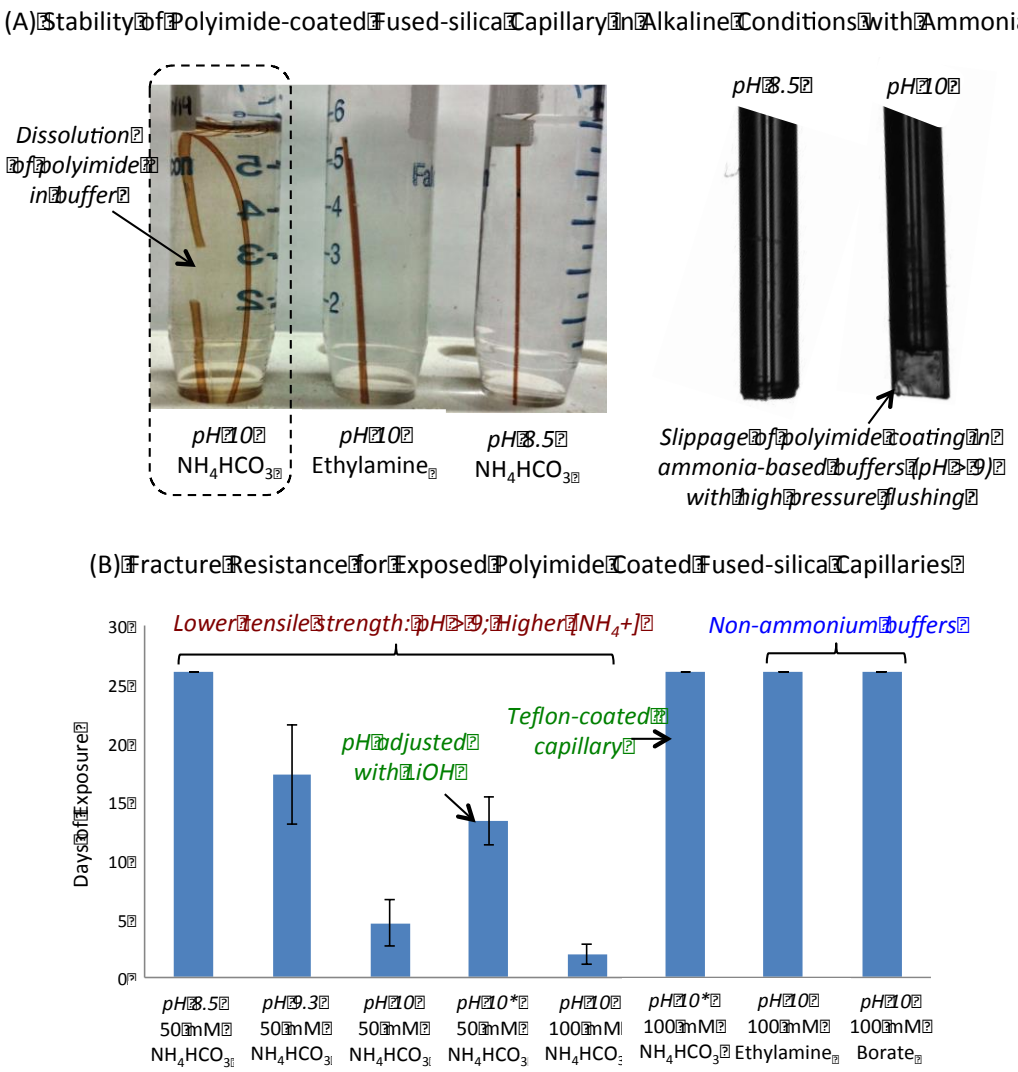

Figure 1. (a) Images depicting the impact of prolonged exposure (70 days) of polyimide coated fused-silica capillaries in aqueous alkaline ammonium buffers that result in softening/deformation of the outer coating and polymer dissolution. High pressure (90 kPa) flushing with 50 mM ammonium bicarbonate buffers for 24 h demonstrate elongation of the outer polymer coating beyond the fused-silica capillary tip at pH 10 that is not observed at pH 8.5 (b) A comparison of changes in the tensile strength and resistance to fractures with repeated bending (90°) to a series of fused-silica capillary segments (where error bars represent ±1s, n=6) exposed to different aqueous alkaline solution (i.*e.*, buffer type, pH, ionic strength), which confirms that higher ammonia concentrations and elevated pH conditions accelerate polyimide aminolysis shortening their average lifespan due to capillary column breakage. This irreversible chemical degradation process is prevented by using ammonium buffers at lower pH (pH 8.5), substituting less nucleophilic or "ammonia-free" alkaline buffers (*e.g.*. borate, ethylamine) or applying more chemically-resistant polytetrafluroroethylene coated fused silica capillaries.

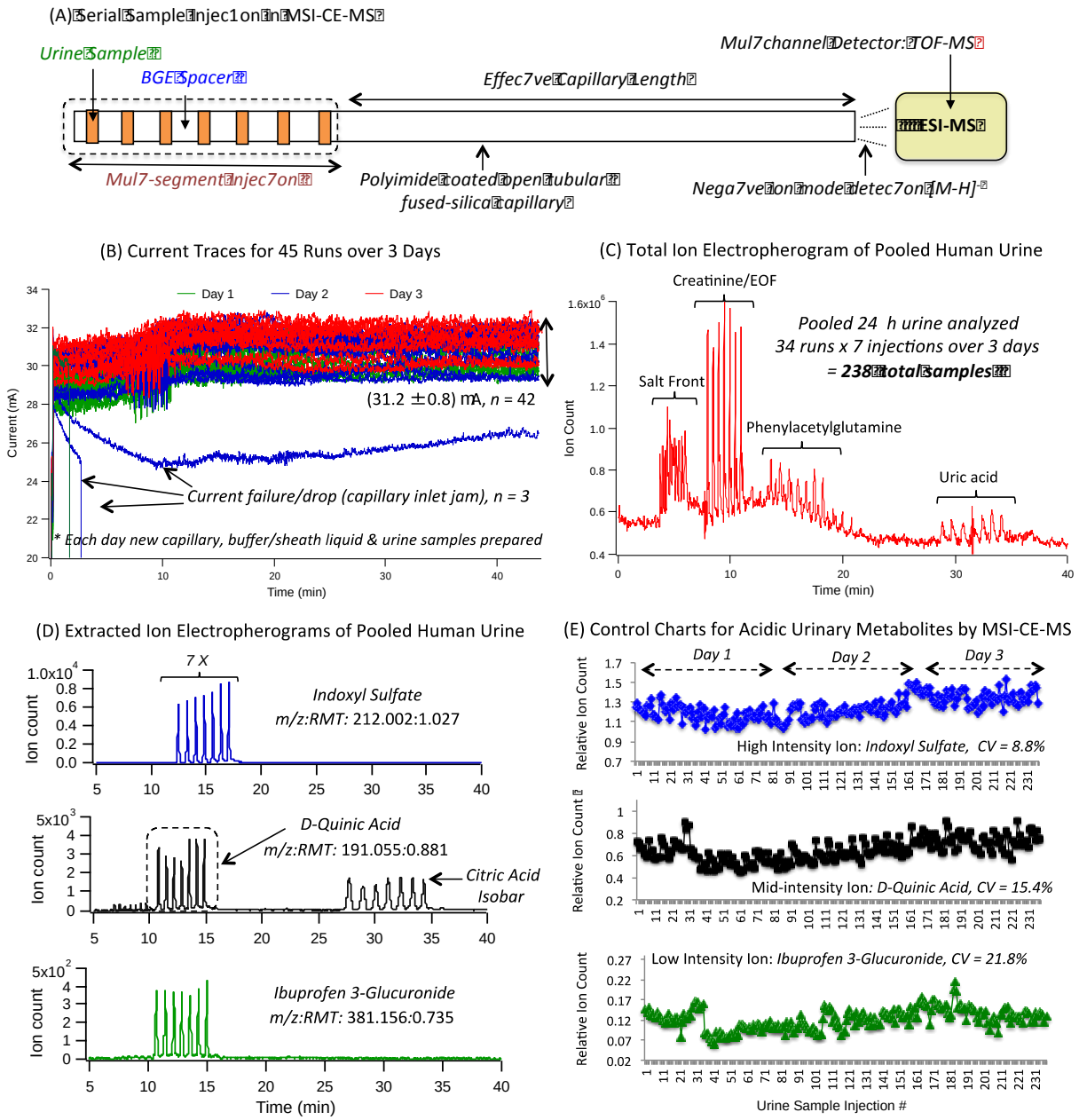

Figure 2. (a) Serial injection format used in MSI-CE-MS for multiplexed analysis of anionic metabolites from seven or more urine samples under alkaline conditions with negative ion mode detection. (b) An overlay of electrophoretic current traces from 45 runs performed over 3 days of analysis, which highlights excellent reproducibility (*CV* = 2.3%) with exception of 3 runs that were rejected due to major current drops caused by incidental capillary inlet misalignment during sample loading. (c) Representative total ion electropherogram (TIE) of MSI-CE-MS when using a 7 serial injection format when analyzing pooled 24 h human urine samples, which illustrates four distinctive transitions during the separation, including a salt front of inorganic electrolytes (*e.g.*, Na^+), neutral compounds co-migrating with the EOF (*e.g.*, creatinine), as well as major urinary metabolites, including phenylacetylglutamine and uric acid. (d) Extracted ion electropherograms (EIE) for three urinary metabolites as defined by their characteristic *m/z*:RMT demonstrating excellent resolution of seven serial injections of human urine samples within a single run with isobaric/isomeric resolution. (e) Control charts highlighting the long-term analytical performance of MSI-CE-MS based on the integrated peak areas for ions relative to an internal standard (NMS) that differ in their natural abundance following 34 runs or 238 repeated injections of a 5-fold diluted pooled human urine sample over 3 days.

Table 1. Long-term stability for analysis of 30 acidic metabolites analyzed in pooled 24 h human urine over three consecutive days of analysis (34 runs or 238 sample injections) by MSI-CE-MS.

Assignment	***m/z*:RMT**	**Molecular formula**	**Mass error *ppm***	**RMT *%CV***	**RPA *%CV***
Lactic Acid	89.0244:1.154	$C_3H_6O_3$	6.0	0.7	14.2
3-Hydroxybutyric Acid	103.0401:1.026	$C_4H_8O_3$	1.3	0.1	17.8
Succinic acid	117.0193:1.950	$C_4H_6O_4$	3.1	1.8	20.6
Oxoproline	128.0353:1.020	$C_5H_7NO_3$	2.3	0.1	13.5
Glutaric Acid	131.0350:1.700	$C_5H_8O_4$	1.4	1.5	20.7
Threonic Acid	135.0299:0.993	$C_4H_8O_5$	1.7	0.1	11.6
Tartaric Acid	149.0092:2.019	$C_4H_6O_6$	0.1	1.8	28.1
4-Hydroxyphenylacetic Acid*	151.0401:0.932	$C_8H_8O_3$	0.3	0.4	14.2
2-Aminoadipic Acid*	160.0615:0.898	$C_6H_{11}NO_4$	0.8	0.6	11.7
3-Hydroxymethylglutaric Acid*	161.0455:1.602	$C_6H_{10}O_5$	0.0	1.6	22.3
Uric Acid	167.0201:0.959	$C_5H_4N_4O_3$	4.2	0.3	13.8
2-Isopropylmalic Acid*	175.0601:1.456	$C_7H_{12}O_5$	5.1	1.3	19.2
Hippuric Acid	178.0510:0.905	$C_9H_9NO_3$	1.7	0.5	10.2
Homovanillic Acid	181.0506:0.880	$C_9H_{10}O_4$	2.8	0.8	9.9
4-Pyridoxic Acid*	182.0459:0.936	$C_8H_9NO_4$	1.3	0.4	18.6
p-Cresol Sulfate	187.0071:1.068	$C_7H_8O_4S$	2.8	0.3	9.3
5-Hydroxyindoleacetic Acid	190.0510:0.880	$C_{10}H_9NO_3$	2.5	0.7	13.5
D-Quinic Acid	191.0552:0.881	$C_7H_{12}O_6$	4.7	0.7	15.4
Vanillylmandelic Acid	197.0552:0.876	$C_9H_{10}O_5$	11	0.8	16.4
Indoxyl Sulfate	212.0023:1.027	$C_8H_7NO_4S$	0.6	0.1	8.8
Pantothenic Acid	218.1034:0.816	$C_9H_{17}NO_5$	0.9	1.1	16.8
Ethyl Glucuronide	221.0746:0.828	$C_8H_{14}O_7$	1.4	1.0	24.2
2-Hydroxy Ibuprofen*	221.1183:0.799	$C_{13}H_{18}O_3$	2.8	1.2	14.2

Acetaminophen Sulfate	230.0129:0.921	$C_8H_9NO_5S$	0.3	0.4	10.3
Indolylacryloylglycine*	243.0771:0.868	$C_{13}H_{12}N_2O_3$	0.6	1.0	17.9
Phenylacetylglutamine	263.1037:0.805	$C_{13}H_{16}N_2O_4$	0.6	1.1	17.1
Acetaminophen Glucuronide*	326.0881:0.757	$C_{14}H_{17}NO_8$	0.2	1.5	22.1
Ibuprofen Glucuronide*	381.1555:0.735	$C_{19}H_{26}O_8$	0.6	1.6	21.8
Tetrahydroaldosterone 3-Glucuronide*	539.2498:0.704	$C_{27}H_{40}O_{11}$	1.9	1.8	20.2
Cortolone 3-Glucuronide*	541.2654:0.700	$C_{27}H_{42}O_{11}$	0.1	1.8	20.5

An asterisk (*) indicates putative identification of urinary metabolites based on their accurate mass and characteristic electromigration behavior using the Onsager-Hubbard equation. Other metabolites were confirmed by spiking urine with authentic chemical standards. Naphthalene monosulfonic acid (NMS) was used as a single non-deuterated internal standard for data normalization in terms of relative peak areas (RPA) and relative migration times (RMT). Mass error was calculated from the average of experimentally measured m/z (n = 18) taken from the first sample run of each day and the theoretical monoisotopic mass from molecular formula.