Capillary electrophoresis mass spectrometry applied to structural proteomics and small molecule analysis

JORDAN AERTS
Abstract

Capillary electrophoresis with mass spectrometric (CE–MS) detection offers a separation method without equal in terms of flexibility, utility, and cost efficiency. Here we demonstrate precisely this through the application of several laboratory-built CE–MS instruments for the separation of brain metabolites in non-primates, enantioselective separations of synthetic anesthetic metabolites in fractionated pony urine, application in structural proteomics workflows, and identification of exogenous alkaloid biotransformation products in human cerebrospinal fluid (CSF).

We outline a method for quickly and affordably etching austenitic steel tubing, which is widely used in electrospray sources for CE–MS. The stainless steel tapered tip emitters provide robust electrospray with low sheath liquid flow rates and can be easily fabricated in-house, offering flexibility and cost-efficiency when commercial options are unavailable. We contribute a CE–MS method for enantiomer separation, specifically targeting 6-hydroxynorketamine (HNK). By introducing chiral selectors into the separation capillary, the method enables efficient enantiomer separation and offers a new tool to assist with research on HNK as a cure for depression.

We explore the feasibility of cold CE–MS in hydrogen deuterium exchange workflows. The utilization of a lab-designed Peltier-cooled CE device achieves deuterium back exchange rates on par with commercial liquid chromatography-based platforms, offering new possibilities for studying protein structures and interactions.

We also demonstrate the wide ranging versatility of CE–MS with contributions to the identification of specific tobacco related metabolites in CSF samples during the development of a high throughput mass spectrometry diagnostic tool for Parkinson’s Disease.

This thesis showcases the versatility and value of CE–MS in various applications, a true blessing for analytical chemistry.

Keywords: Capillary Electrophoresis Mass Spectrometry, Hydrogen Deuterium Exchange Mass Spectrometry, Enantioselective Separations, Drugs of Abuse

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ISSN 1651-6192
URN urn:nbn:se:uu:diva-512502 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-512502)
Dedicated to all hard-working doctoral students at Uppsala University
List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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# Abbreviations

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<tr>
<td>ACN</td>
<td>Acetonitrile</td>
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<tr>
<td>BE</td>
<td>Back-Exchange</td>
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<td>BFS</td>
<td>Bare Fused Silica</td>
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<td>BGE</td>
<td>Background Electrolyte</td>
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<td>BUP</td>
<td>Bottom-Up Protein Analysis</td>
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<td>CCS</td>
<td>Ion-Neutral Collision Cross-Section</td>
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<td>CD</td>
<td>Cyclodextrin</td>
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<tr>
<td>CE</td>
<td>Capillary Electrophoresis</td>
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<td>CSF</td>
<td>Cerebrospinal Fluid</td>
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<tr>
<td>DHK</td>
<td>Dehydroketamine</td>
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<td>DHNK</td>
<td>Dehydroxynorketamine</td>
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<tr>
<td>DIA</td>
<td>Data-Independent Acquisition</td>
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<td>EOF</td>
<td>Electroosmotic Flow</td>
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<tr>
<td>ESI</td>
<td>Electrospray Ionization</td>
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<td>ETD</td>
<td>Electron Transfer Dissociation</td>
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<tr>
<td>Hb</td>
<td>Bovine Hemoglobin</td>
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<td>HDX</td>
<td>Hydrogen Deuterium Exchange</td>
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<tr>
<td>HEPES</td>
<td>N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>HK</td>
<td>Hydroxyketamine</td>
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<tr>
<td>HNK</td>
<td>Hydroxynorketamine</td>
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<tr>
<td>HPLC</td>
<td>High-Performance Liquid Chromatography</td>
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<td>ID</td>
<td>Inner Diameter</td>
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<td>IM</td>
<td>Ion Mobility</td>
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<td>KET</td>
<td>Ketamine</td>
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<td>Acronym</td>
<td>Description</td>
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<tr>
<td>LC</td>
<td>liquid chromatography</td>
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<td>LIF</td>
<td>Laser-Induced Fluorescence</td>
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<tr>
<td>LPA</td>
<td>Linear Polyacrylamide</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix Assisted Laser Desorption/Ionization</td>
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<tr>
<td>MCE</td>
<td>Microchip Capillary Electrophoresis</td>
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<tr>
<td>MNK</td>
<td>Methoxynorketamine</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
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<tr>
<td>MS(^E)</td>
<td>All Ions Fragmentation</td>
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<tr>
<td>MT</td>
<td>Migration Time</td>
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<td>(m/z)</td>
<td>mass-to-charge ratio</td>
</tr>
<tr>
<td>NCot</td>
<td>Norcotinine</td>
</tr>
<tr>
<td>Nic</td>
<td>Nicotine</td>
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<tr>
<td>NK</td>
<td>Norketamine</td>
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<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance Spectroscopy</td>
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<tr>
<td>OD</td>
<td>Outer Diameter</td>
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<tr>
<td>PD</td>
<td>Parkinson’s Disease</td>
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<tr>
<td>PFT</td>
<td>Partial Filling Technique</td>
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<tr>
<td>SL</td>
<td>Sheath Liquid</td>
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<tr>
<td>SS</td>
<td>Stainless Steel</td>
</tr>
<tr>
<td>ToF</td>
<td>Time of Flight</td>
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<tr>
<td>TRIS</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TTS</td>
<td>Triple Tube Sprayer</td>
</tr>
<tr>
<td>UDMS(^E)</td>
<td>UltraDefinition MS(^E)</td>
</tr>
<tr>
<td>UPLC</td>
<td>Ultra-Performance Liquid Chromatography</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet Light</td>
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Introduction

The Evolution of Electrophoresis
The story of electrophoresis begins in Sweden. In 1948 Arne Tiselius was awarded the Nobel Prize in Chemistry partly for his work on electrophoresis [1]. Regarded as the father of electrophoresis, Tiselius, in his Nobel Lecture, describes the phenomenon as “the migration of substances in an electric field”, though he acknowledges previous work on proton migration in a tube of phenolphthalein jelly by Oliver Lodge in 1886, as well as the migration of proteins in U tubes presented by Hardy between 1899 and 1905. Tiselius’ own work in electrophoresis began in 1925 in Uppsala, Sweden when he began working on a project started by his PhD advisor, Theodor Svedberg.

In the days since Tiselius, electrophoresis has been applied mainly for the analysis of molecules that can carry a charge and is currently used primarily in the fields of biological and biochemical research, protein chemistry, pharmacology, forensic medicine, clinical investigations, veterinary science, molecular biology, and food control [2]. Three different electrophoretic principles are used for the separations: zone electrophoresis, isotachophoresis, and isoelectric focusing. All work described in this thesis was performed in the zone electrophoresis regime. The work presented here was possible due to the maturation of electro-migration techniques over the course of the last 135 years.

The development of capillary electrophoresis (CE) again begins in Sweden. Tiselius and his graduate student, Stellan Hjertén began publishing between 1965-1967 zone electrophoretic separations in 3 mm inner diameter (ID) capillaries [3]. The impetus for Hjertén’s drive towards miniaturization was that he found it difficult to find 100 mg of a protein which was necessary for the electrophoretic separations being conducted by Tiselius and this resulted in separations in capillaries with IDs as small as 5 µm [4].

Though this first work in CE was published in the late 1960s, much credit for the development of capillary zone electrophoresis is given to James Jorgensen, for his work in the field in the early 1980s [5–8]. Eight years after Jorgensen and Lukacs’ 1981 thorough evaluation of zone electrophoresis in open tubes, the first commercial CE instrument was released [9]. Though this helped to advance the field of CE, by this time high-performance liquid chromatography (HPLC) instruments had already been commercially available for more than 12 years [10].
Capillary Electrophoresis

Standard format CE occurs in a circuit such as shown in Figure 1 which is comprised of a fused silica capillary with an outer coating of polyimide (\(\sim 15\) µm thick), filled with a conductive background electrolyte (BGE) with an anode and cathode at either end making electrical contact through the BGE filled capillary. This basic circuit is at the heart of today’s commercially available instruments such as the PrinCE Next 800 series, Promega Spectrum, Agilent 7100, and Sciex P/ACE MDQ plus, GenomeLab GeXP, PA 800 Plus, BioPhase 8800 and CESI 8000 plus systems [11–13].

**Figure 1.** Pictorial diagram of a standard capillary electrophoresis circuit.

CE affords high efficiency, reproducible separations due to several well characterized phenomena. In CE the flow profile is flat, unlike the parabolic flow profile of pressure driven systems (HPLC, ultra-performance liquid chromatography (UPLC)). This is the result of electroosmotic flow (EOF). Because the capillaries used are made from fused silica, and filled with a liquid buffer, at pH >3, the silanol groups (\(-\text{SiOH}\)) at the capillary inner lumen will dissociate to become negatively charged silanate ions (\(-\text{SiO}^-\)) [1]. These silanate ions will then attract cations from solution to form a stagnant layer called the Stern layer, which will itself attract mobile solvated cations creating a diffuse...
layer (Gouy-Chapman layer) in a concentration gradient across a distance of several hundred nanometers until the distribution of solvated cations matches that of the bulk solution [14]. The concentration gradient created by the ions across the stagnant and diffuse layers generates what is called the zeta potential, \( \zeta \), and is described by the Smoluchowski formula:

\[
\zeta = \frac{4\pi \eta \mu_{eo}}{\varepsilon}
\]

where \( \eta \) is the viscosity, \( \mu_{eo} \) is the coefficient for EOF, and \( \varepsilon \) is the dielectric constant of the solution [9]. To achieve the separations in CE, a large electric field is applied across the capillary and as a result, the cations of the diffuse layer will migrate toward the cathode, transporting the bulk solvent with it.

EOF has a linear velocity, \( v \), given by:

\[
v = \frac{\varepsilon}{4\pi \eta} E \zeta
\]

where \( E \) is the field strength (\( V/L \)), \( V \) being the voltage applied across the capillary, \( L \) being the length of the capillary [15, 16]. EOF originates at the surface of the capillary and can be impacted by BGE additives and analyte adsorption [17]. EOF will provide a plug-like flow profile so long as the capillary inner radius is greater than 7 times the thickness of the double layer [18].

All of the CE separations presented in this thesis were performed in anode to cathode mode. Under this regime, cationic analyte mobilities are in the same direction of the EOF (coelectroosmotic migration) and anionic analyte mobilities are directed in the opposite direction (counterelectroosmotic migration) while neutral analytes will have a migration velocity equal to the EOF. The separation of cations, anions, and neutrals from each other is determined by the electrophoretic mobility, \( \mu_{ep} \), of the analyte, and can be calculated by:

\[
\mu_{ep} = \frac{q}{6\pi \eta r}
\]

where \( q \) is the charge and \( r \) is the radius of the analyte. Analyte migration velocity in an electric field is given by [9]:

\[
v = \mu_{ep} E = \frac{\mu_{ep} V}{L}
\]

The apparent mobility, \( \mu_{app} \), of an analyte then will be a combination of \( \mu_{eo} \) and \( \mu_{ep} \) [15]:

\[
\mu_{app} = \mu_{eo} \pm \mu_{ep}
\]
The primary source of band broadening in CE then is longitudinal diffusion as the separation is conducted in a single phase of uniformly flowing carrier liquid [19]. Briefly, diffusion can be exacerbated by convective mixing which can be minimized by using smaller ID capillaries which offer greater surface area to volume ratios to allow for joule heat dispersion through the capillary walls. The positive impact of thermostatting capillaries is also documented and all commercial CE instruments provide cooling capabilities to improve migration time (MT) reproducibility and minimize band broadening [20, 21].

Sample Injection

Due to the small volume inside of the capillary CE has historically suffered from the ability to inject only pL to nL amounts of sample [22]. Introducing the sample into the capillary can be achieved via two routes, both of which begin by temporarily placing the inlet of the capillary into the liquid sample. The amount of sample injected is then determined by manipulating the pressure at the capillary inlet (hydrodynamic injection), or by applying a moderate electrical potential (electrokinetic injection) through the liquid sample, driving the charged analytes into the capillary and towards the oppositely charged electrode [19]. Because the injection is biased towards more highly charged species, one must be aware that the composition of the sample plug introduced with electrokinetic injection is not representative of the original sample solution.

Commercial CE instruments are capable of performing both types of injections as the electrodes sit in parallel with and several mm from the separation capillary inlet/outlet. When the capillary inlet is inserted in the sample vial, the electrode enters the vial as well and a gas tight seal is produced, allowing pneumatic pressure or vacuum to be applied into the sample vial. Typically the electrode is placed a few mm higher than the inlet of the capillary as introducing it into the liquid sample presents the opportunity for contamination of the electrode surface, though if electrokinetic injection is desired, the electrode must also enter the sample solution.

Hydrodynamic injection can be performed by pressure, vacuum, or the generation of a gravity flow [22]. In experiments conducted for Papers I, II and IV, gravity flow injection was achieved by inserting the capillary inlet into a stainless steel (SS) vial whence several μL of sample solution was deposited. The sample vial was located on an acrylic platform as shown in Figure 2 and the capillary inlet was placed in the liquid sample droplet, the platform was then elevated to place the capillary inlet 13 cm higher than the capillary outlet, and left in place for 60-180s to produce the gravity flow, then the platform was returned to the original position and the capillary inlet returned to the BGE vial.
and the separation initiated. The volume of sample injected, $S_{inj}$, is given by:

$$S_{inj} = \frac{\rho g \pi r^4 \Delta h_i}{8 \eta L}$$

(6)

where $\rho$ is the density of the sample solution, $g$ is the gravitational acceleration constant, $r$ is the capillary inner radius, and $\Delta h$ is the height difference between the liquid levels at the inlet and outlet of the capillary [22]. Detailed pictures of a similar injection platform can be seen in panels a and b, figure 1 in ref [23].

**Figure 2.** Pictorial diagram of a simple hydrodynamic injection platform. (A) XYZ capillary inlet positioning block. (B) XY capillary inlet positioning block. (C) Sample vial. (D) Stainless steel BGE vial housed in a stainless steel block which is connected to the load of a high voltage power supply. (E) Capillary inlet. (F) Capillary continues towards mass spectrometer inlet where it terminates at the outlet of a coaxial sheath flow electrospray emitter (shown in Figure 3).

Detection Regimes

Though all of the work presented in this thesis uses mass spectrometry (MS) as the detection method, there exist several other detection methods which warrant a brief discussion.
Electrochemical Detection
Despite the requirement for isolating the detection system from the large voltage drop across the separation capillary, amperometric, potentiometric and conductivity detection schemes have been employed with successful detection of sub attomole quantities of analytes [9].

Light Based Detection
Because fused silica does not absorb light between ∼180 nm and 3500 nm, on-column detection of analytes using ultraviolet (UV) and visible light absorbance as well as laser-induced fluorescence (LIF) has dominated CE separations, while refractive index and raman spectrometric detection have also been incorporated [24]. While many compounds inherently absorb UV light, most are not intrinsically fluorescent, creating the need for pre- or post-column derivatization to introduce fluorophores to the analyte molecules [9].

Indirect detection can also be used for detection of analytes that do not themselves elicit a response in CE–LIF and CE–UV. For indirect detection, a chromophore or fluorophore is added to the BGE, providing a constant signal to the detector [25]. When a highly concentrated analyte zone migrates past the detector the fluorophore/chromophore will be displaced, resulting in a decrease of the background signal.

Mass Spectrometry Detection
Capillary electrophoresis has utilized MS detection of several varieties. A more detailed explanation of MS is provided in a later section. When using MS detection for CE separations, the CE circuit is typically modified similar to Figure 3, though the coaxial sheath flow electrospray ionization (ESI) emitter shown is not the only option for coupling CE to MS. The illustration shows the circuit specifically used for an MS instrument which has the inlet as ground (e.g. Waters MS), for instruments which apply the ESI voltage to the MS inlet (e.g. Bruker MS), the CE–MS emitter will be connected to earth ground and no ESI voltage will be applied to the emitter itself. Similar CE circuits can be found in in CE-MS specific instruments such as the Sciex CESI 8000, CMP Scientific ECE-001, and EMAST-A, and the microchip CE (MCE) based ZipChip from 908 devices, though in most cases commercial CE–UV/LIF instruments can be modified to operate in this mode using the appropriate commercial or laboratory-built CE–MS interfaces discussed below [11, 13, 26, 27].

The requirement for the CE circuit modification is due the need to generate gas phase ions from the CE effluent by ESI for MS detection while simultaneously completing the CE electrical circuit in the absence of an outlet buffer vial. The primary disadvantage of MS coupling is that the favorite BGE additives used in CE, borate and phosphate, are not volatile and thus incompatible
Figure 3. Pictorial diagram of a normal polarity CE circuit with coaxial sheath-flow ESI using MS detection where the MS inlet is ground. (A) High voltage DC power supply. (B) Cathode connection to stainless steel needle of the sheath liquid capillary. (C) Syringe pump for delivering sheath liquid. (D) Sheath liquid capillary. (E) Mass spectrometer inlet. (F) Stainless steel hypodermic tubing used as electrospray emitter and receiving electrospray voltage from the MS. (G) Polyether ether ketone tee connector allowing for coupling the separation capillary to the electrospray emitter while incorporating a coaxial flow of conductive sheath liquid around the separation capillary to both complete the electrical circuit for CE and facilitate ESI. (H) Separation capillary. (I) Anode connection to metal block holding BGE vial.

for routine use with online MS detection. ESI is thought to occur slightly differently depending on the size of the analyte, but the three common steps that occur to a conductive liquid in an applied electrical gradient are the creation of fine droplets followed by desolvation of these droplets, subsequent Coulombic repulsion which generates smaller daughter droplets and finally the release of gas-phase ions [28–30]. Shown in Figure 4 is a photomicrograph of the ESI generated with the experimental conditions used in Paper II.

In 1987 an online ESI interface for CZE was first reported, and evolved into a tube within a tube within a tube (triple tube sprayer, TTS) for delivering the separation capillary at the center, surrounded by a tube carrying sheath liquid (SL) which is then jacketed by a tube carrying nebulizing gas [31, 32]. In this arrangement, the SL is used to complete the electrical circuit needed
Figure 4. Image of cone jet regime of ESI used for all investigations in this report.

for the CE separation. A commercially available TTS sprayer was first offered by Hewlett Packard in 1995, and it is still available from Agilent today [33]. This TTS is a bit cumbersome, and even though it works well, it requires µL/min SL flow rates, leading to severe dilution of the CE effluent in combination with the need for a nebulizing gas to facilitate droplet generation from such high SL flow rates. Combined, the siphoning effect of both the SL and nebulizing gas produce parasitic flow contributions which can be detrimental to the CE separations and sometimes requires significant optimizations to the ESI parameters (ES voltage, SL composition/flow rate, capillary protrusion length, nebulizing gas flow rate, and sprayer distance from MS inlet) when different BGEs are used [30]. In light of the disadvantages of early CE–MS interfacing, sheath flow interfaces have progressed into nL/min flow rates and subsequently, sheathless interfaces in which there is no dilution of the CE effluent. The TTS has been used in the nL/min flow regime by the omission of nebulizing gas, but an alternative sprayer utilizing 10s of nL/min sheath flows has been commercialized by CMP Scientific based on an electrokinetically pumped SL interface out of the Dovichi lab [26, 34].

The most popular sheathless CE–MS interface is based on the work of Moini and involves the use of a separation capillary with an outlet etched by
hydrofluoric acid to the point that the capillary wall has achieved a thickness of 5-10 μm which allows for the passage of ions through the glass wall [35]. This interface has been commercialized by Sciex where it provides the interface for the CESI 8000 series of CE instruments [13]. The porous tip sheathless interface features the advantages that there is no SL flow, though several mm from the capillary outlet the porous region of the capillary is in contact with a conductive liquid for CE circuit completion, and therefore any generation of gas bubbles by electrolysis of SL will not disrupt ionization of the CE effluent, but a main drawback of the interface is that the BGE must not only provide CE separations, but also support stable ESI, and thus the choice of BGEs is further limited vs CE–MS using sheath flow interfaces [30]. The sheathless sprayers from Sciex conveniently come pre-etched and assembled in a cartridge which plugs right into the CE instrument, and is available with several coatings (linear polyacrylamide (LPA), polyethylenimine) for enhanced proteomics capabilities, but they are not cheap at around $2,000 each [13]. The above mentioned CE–MS interfaces are simply the most popular in the field at the moment and do not comprise an exhaustive list of the options available, including the coaxial sheath flow nanoelectrospray emitter used for the investigations in this report [23].

Non-ESI CE–MS Interfacing
Offline fractionation, or continuous spraying of CE effluent onto metal targets using spotting robots in conjunction with matrix assisted laser desorption/ionization (MALDI) MS detection has been used [36]. Offline analysis with MALDI MS has three major benefits over online MS detection: a higher tolerance for non-volatile buffer additives, reduced constraints for high instrumental duty cycles to be able to match the narrow analyte peaks generated by CE separations, and in the case of peptide analyses, very low detection limits [37].

Another CE–MS interface of note allows for the coupling of CE to an inductively coupled plasma ionization source. In this case, the CE effluent is nebulized into a fine aerosol which passes through a cooled spray chamber, removing the largest droplets, and is then fed into a 6000 K neutral argon plasma which dries the aerosol, decomposes the sample, and strips an electron from the atoms, generating monoatomic cations at a rate described by the Saha equation for introduction into the MS [38]. What this method of ionization allows for is the quantitation of individual elements in a sample, and in combination with CE, provides, for example, a method for distinguishing between harmless organic species of arsenic and selenium (e.g., arsenobetaine, arsenocholine, selenocysteine and selenomethionine) from their carcinogenic inorganic counterparts (As(III), As(V), Se(VI), and (SeIV)), an application highly sought after in agriculture, food products, and medical industries [39–42]. One major advantage of this harsh ionization technique is that BGE com-
position has no impact on ionization and thus the standard phosphate buffers can be employed.

Capillary Coatings
Depending on the analytes one wishes to separate, masking of the silanol groups of the capillary inner lumen might be desirable or necessary [43, 44]. Beginning in 1985, the advantages of masking the silanol groups by a covalent linkage to polyacrylamide had been observed to eliminate solute adsorption to the capillary lumen [45]. Capillary coatings are mainly implemented to eliminate analyte adsorption to the capillary wall, or to modify the EOF. By applying a capillary coating one can eliminate, increase, decrease, or reverse EOF, though the change in EOF may or may not be stable across all pH values [43].

Dynamic coatings are BGE additives which competitively inhibit analyte adsorption to the silanol or silanate groups on the capillary wall, and are typically incompatible with MS as they can cause ionization suppression and MS source contamination. Static coatings can be adsorbed to the capillary wall or covalently bound via silane chemistry and polymerization reactions. To provide reproducible separations and prevent analyte adsorption, a static coating must be homogenous and cover the entire surface of the capillary, provide steric hindrance, electrostatic repulsion, or surface inertness to prevent analyte adsorption to the silanol groups, and be stable at the pH of the BGE to prevent bleeding into the MS [43].

Manipulation of EOF is not without consequence as some amount of flow is absolutely necessary when using the sheathless CE–MS interfaces discussed below, but also at sufficient velocity and especially upon EOF reversal, the penetration of ions from the SL into the separation capillary has the potential to change BGE pH and disrupt the separation, in some cases requiring pressure assistance for successful separations [46, 47]. A balance must be reached with the capillary coating and the intended outcome of the separations as increasing EOF will simultaneously decrease resolution and MTs for analytes with coelectroosmotic migration, and increase the resolution of capabilities for separating analytes having counterelectroosmotic migration [48].

In Papers I–IV, bare fused silica (BFS) capillaries were used without coating. In Paper III both BFS and LPA coated capillaries were employed. We used a modified method for producing the LPA coating [49]. The LPA coating has a combined effect of preventing protein and peptide adsorption to the capillary wall as well as eliminating EOF. High quality peptidomics studies have been conducted using LPA coated capillaries, with the noted result that
the coverage of hydrophilic peptides is increased using CE–MS compared to UPLC [50].

Mass Spectrometry
The successful application of MS is dependent on three fundamental processes working in conjunction: Ion generation, ion transmission, and ion detection.

Ionization
Ionization regimes will be determined by the analytes of focus in the mass spectrometric investigation. For analysis of individual elements all chemical bonds must be broken, requiring harsh plasma ionization at temperatures hotter than the surface of the sun (6500 K) [38]. More gentle ionization methods include bombarding the surface of a sample with a primary ion beam of C$_{60}^+$ buckyballs or gas clusters such as (CO$_2$)$_{6000}^+$ or H$_2$O$_{1000}^+$ such that a series of collision cascades can eject intact molecular species from the top two or three monolayers of the sample, some of this sputtered material being charged secondary ions which the MS can detect [51]. Less traditional ionization methods such as shooting a metal plate coated with protein standards with a pellet from an air rifle have also been reported [52]. As discussed in the previous section on detection regimes in CE, we have used ESI for ion generation, though sometimes we have used CE to support analyses using MALDI–MS, thus a brief explanation of MALDI is warranted. Small organic acids or bases (matrix) that strongly absorb light can be applied to various samples such as thin sections of intact tissue, homogenized tissues, and bio fluids resulting in crystallized analyte-matrix amalgam which can be irradiated with an appropriate wavelength laser pulse. The irradiation excites the matrix molecules resulting in desorption from the tissue or sample surface and subsequent ionization of the molecules co-crystalized with the matrix. Sometimes a drawback of MALDI is that complex samples such as tissue sections will result in many ions being generated and the identification of a single feature can be difficult depending on the presence of background ions or high abundance ions of similar mass. For a more detailed historical treatment of MS and ionization techniques, the reader is recommended to read Ref [53].

Ion Transfer
Many combinations of ion optics are used inside of mass spectrometers to transfer the generated ions from the ion source to the detector. Traditionally the ion path is comprised of metal ion guides carrying direct current and radio frequency voltages to filter out ions outside of the range of interest for the
study and transmit the ions of interest to a mass analyzer where they are separated based on their mass-to-charge ratio \( (m/z) \). Most MS instruments also contain right angles in the ion path to assist in keeping neutrals from contaminating the ion optics and also to prevent photons from reaching the detector.

Residual gas molecules in the ion optical path have a deleterious effect in MS so the first step in most MS instruments is bridging (mostly) atmospheric pressure (760 Torr) ionization in the source of the instrument with increasing vacuum pressures inside of the mass spectrometer. The first vacuum stage is called rough vacuum and is \( 10^{-3} \) Torr. At the terminus of the ion optical path, where the detector resides, vacuum pressures will be anywhere from \( 10^{-5} \) to \( 10^{-9} \) Torr. The high vacuum accomplishes three things: it prevents signal loss from the collision of analyte ions with background gases by creating a long mean free path, it creates a chemically clean environment to prevent gas phase chemical reactions between the analyte ions and background gas, and it allows the operation of electrical components in the MS to operate at high voltages without electrical arcing [54].

More recently many MS instruments feature ion mobility (IM) cells along the ion path and as a result are able to perform gas phase separations and provide additional ion-neutral collision cross-section (CCS) information on the ions traveling through the MS. The specific instrument used in this work was a Synapt G2-Si from Waters Corporation. The ion optics of the Synapt are shown in Figure 5. There are multiple options for mass analyzers, such as quadrupole, magnetic sector, ion trap, Fourier-transform ion cyclotron resonance (FT–ICR), and time of flight (ToF) [55]. The Synapt uses quadrupole and ToF mass analyzers and like other ToF instruments it is a good match for the narrow analyte peaks produced by CE separations due to the high duty cycle of the ToF analyzer. The Synapt has a maximum acquisition rate of 30 Hz in ToF mode, and 10Hz when IM is enabled [56].

The Triwave cell in the ion optical path, contains a traveling wave IM cell seen in a close up view in Figure 6. The IM cell allows for the separation of ions through an inert gas within a weak electric field according to their CCS \( (\Omega) \) and with careful measurements of calibrant molecules of known \( \Omega \) \( \text{(in } \text{Å}^2) \) and \( m/z \), the physical size of an ion can be calculated from its measured drift time through the mobility cell [58].

**Tandem MS**

One of the features of certain mass spectrometers is the ability to isolate mass-selected (precursor) ions with the first mass analyzer and subject them to sufficient energy through collisions with an inert gas in a collision cell thereby producing fragments of the precursor ions, called product ions. This tech-
Figure 5. Schematic illustration of the ion optical path components in the Synapt G2-Si MS used in the investigations in this report [57].

**Triwave technology:**

Figure 6. Schematic illustration of the ion mobility component in the Synapt G2-Si MS used in the investigations in this report [57].

The technique of tandem MS is called collision induced dissociation (CID). When you already know the $m/z$ of the precursor ions in your experiments, a mass list is
entered into the MS software to select specific m/z at specific MTs to perform CID fragmentation. This method of MS/MS is called data-dependent acquisition. By matching the pattern of ion fragments to those generated from a standard compound, or to an online database, you can determine with a high degree of certainty the identity of the precursor ion.

In situations where you want to generate tandem MS data for as many precursor ions in your sample as possible a brute force method called data independent acquisition (DIA) is used. For Waters instruments this approach is called MS\textsuperscript{E} or “all ions fragmentation”. In Paper III we used IM drift time optimized CID collision energies for enhanced DIA tandem MS, refered to as Ultra Definition MS\textsuperscript{E} (UDMS\textsuperscript{E}), for fragmentation of the peptides separated by CE [59]. How this works is that in IM mode on the Synapt the Triwave trap cell on the left in Figure 6 acts as a gate to release a packet of ions into the mobility cell (IMS T-Wave), in sync with the gated release, the pusher shown in Figure 5 accelerates ions arriving from the IM cell into the ToF analyzer over the course of 200 pushes [57]. 200 pushes into the ToF analyzer constitutes one mobility experiment. So the arrival time distribution of ions passing through the IM cell has been separated into 200 bins in the mobility dimension and what UDMS\textsuperscript{E} does is then targets the early (bins 0-20), middle (bins 20-110), and late (bins 110-200) IM bins for different CID collision energy ramps in the transfer cell (Transfer T-Wave, Figure 6) to enhance precursor fragmentation efficiency over previous MS\textsuperscript{E} approaches [59]. Waters corporation uses a proprietary software ProteinLynx GlobalSERVER (PLGS) to process MS\textsuperscript{E} raw data to provide peptide sequence information. The Synapt used in this thesis is also equipped to perform less commonly used tandem MS fragmentation process called electron transfer dissociation (ETD).

Detection

All mass spectrometers besides FT–ICR instruments require an ion detector [60]. Current instruments typically use Faraday cups, electron multipliers or photo-multipliers to amplify the current of an ion impacting the detector surface via dynodes into a measurable current which can be recorded [61].

Select Applications of CE–MS

With CE–MS, the applications are wide reaching, however, I will only discuss applications of CE–MS directly related to the present investigations discussed in this thesis. These applications are small molecule analysis, separation of chiral compounds, and peptide separations.
Small Molecule Analysis by CE–MS
Metabolomics research is concerned with the comprehensive characterization of the low molecular weight metabolites present in biological systems [62]. CE–MS for metabolomics is notably efficient for profiling polar and charged metabolites such as amino acids, nucleotides, small organic acids and sugar phosphates [63]. A recently published interlaboratory investigation involving the separation of a mixture of 23 cationic metabolites on 20 different CE–MS platforms across 17 different laboratories demonstrated the acceptable reproducibility of the separations and stands as an example of the increased adoption of this once niche technique [64]. CE–MS has been used frequently in single cell and subcellular metabolomics investigations due in part to the fact that the scale of CE (capillary IDs on the scale of µm, injection volumes pL–nL) is similar to the scale of individual cells themselves and is thus able to minimize dilution of the samples [23, 65].

Chiral Analysis by CE–MS
Isomers are molecules having the same chemical formula, but can differ in either connectivity (constitutional isomer) or spatial orientation (stereoisomer) and in the context of MS they can present a hurdle to molecular identification in that generally the readout from a mass spectrometer is an m/z. Constitutional isomers differ in the binding of their atoms in three-dimensional space, an example is shown in Figure 7. Tandem MS can differentiate between constitutional isomers, but having a separation step before introduction to the MS (alternatively IM within the MS) may be necessary if multiple isomers are present in the original sample. Stereoisomers are compounds with identical molecular formulas and sequence of bonds but having differences in their three-dimensional structures [66]. A chirality center occurs when a carbon atom has four different groups bound to it, and this can generate a non-superimposable mirror image, called an enantiomer [67]. For a given molecule, each enantiomer will rotate plane polarized light in the opposite direction, but otherwise have identical physicochemical properties and cannot be separated in achiral media. For molecules with more than one chiral center (n chiral centers), the maximum possible number of stereoisomers is 2^n.

Figure 7. Constitutional isomers octopamine and dopamine.
where \( n \) of those will be enantiomers and the rest will be diastereomers (non superimposable non mirror images). An example is shown in Figure 8 for 6-hydroxynorketamine (HNK) which has 2 stereocenters (indicated by the stars) to demonstrate that the molecule exists as \( 2^2 = 4 \) stereoisomers.

Analytical methods which can perform the separation of enantiomers have attracted great interest since qualitative and quantitative differences in pharmacological activity can exist between enantiomers to the point that the pharmacologically inactive enantiomer may exhibit unwanted or even toxic side effects [68]. Chiral analysis by CE offers a cost advantage and increased flexibility over other chiral analyses due to the fact that only minuscule volumes of BGE additives are needed in CE whereas expensive enantioselective stationary phases are needed for chiral separations by liquid chromatography (LC) and supercritical fluid chromatography. Revisiting the tenet that non-volatile buffer additives are detrimental to CE separations, the common approach to using non-volatile chiral selectors in the BGE is to fill only a part of the capillary with the selector so that it will not foul the mass spectrometer. This partial filling technique (PFT) which we used in Paper II, was pioneered in a CE–UV study that used a high UV absorbing chiral selector which impaired the detection of \( \beta \)-adrenergic receptor blocking drugs [69]. A common BGE additive used for chiral separations by CE are cyclodextrins (CD). CDs are composed of 6 (\( \alpha \)), 7 (\( \beta \)), or 8 (\( \gamma \)) glucose monomers connected by \( \alpha-(1,4)-\)
glucosidic bonds. Shown in Figure 9 are the three cyclodextrins where one can see the presence of a central cavity which is the same depth for each CD but differs in width. Inclusion of a bulky hydrophobic moiety of an analyte into the cavity and interaction of analyte polar groups with hydroxy groups at the mouth of the CD are the basis for chiral recognition [70]. Derivatization of hydroxy groups on the CDs allows for manipulation of charge/solubility and cavity depth of the CDs [70].

![Figure 9. Native α-, β-, and γ- cyclodextrins.](image)

Proteomics by CE–MS
Proteomics aims to describe the full complement of proteins in cells based primarily on their expression, post-translational modifications, interactions, location, and turnover [71]. The most common applications in MS based proteomics involves taking a protein(s) of interest, subjecting them to a proteolytic digestion step and then analyzing the generated peptides, with or without a separation step, with MS, where tandem MS measurements of the peptides provides primary sequence information. This approach is referred to as “bottom-up” protein analysis (BUP).

After the first applications of CE–MS for the analysis of intact proteins and tryptic digests of proteins in 1999, CE–MS has evolved into a powerful tool in the field of proteomics [72, 73]. Proteomics analysis by CE–MS has been conducted for mass limited biological samples, characterization of monoclonal antibodies and antibody-drug conjugates, phosphoproteomics, as well as quantitative screenings of urinary markers of cancers, and kidney and cardiovascular disease [74]. Similar to the interlaboratory CE–MS metabolomics investigation, a collaborative study on the robustness and portability of a CE–MS peptide mapping study was conducted across 13 independent laboratories from academia and industry. The study looked at the separation of bovine serum albumin digested with trypsin in six replicate injections repeated on 2 consecutive days in each lab using a combination of Agilent and Sciex CE instruments, and a combination of CE–MS interfaces and found a reproducibility
of between 0.14 and 1.3% RSD for ten representative peptides on polyvinyl alcohol coated capillaries [75].

Hydrogen Exchange

In 1954 in Copenhagen, Denmark, Aase Hvidt and her boss Kaj Linderstrøm-Lang used density measurements to monitor the exchange of pig insulin peptide backbone amide hydrogens with deuterium, noting that the structured regions of native insulin provided protection from hydrogen exchange [76]. Twenty five years later, Rosa and Richards subjected ribonuclease S protein to tritium exchange at physiological conditions, followed by quenching of the exchange reaction, protease digestion, and HPLC separation of the generated peptides, allowing them to localize peptide backbone amide exchange using nuclear magnetic resonance spectroscopy (NMR) to two peptides of length 8 and 12 amino acids [77]. This workflow has been the standard method for performing hydrogen exchange experiments for structural biological inquiries. In the years since, tritium exchange has been replaced with deuterium exchange and MS has replaced NMR as the preferred detection method.

Contemporary hydrogen deuterium exchange (HDX)–MS experiments primarily focus on a BUP approach where a protein in a physiological solution is diluted into a deuterated environment at physiological conditions for an amount of time before peptides are generated and separated using conditions where the exchange rate is slow, called quench conditions (i.e., pH 2.5 and 0°C) [78]. During the separation step, amino acid side chain residues, exposed to a protiated solvent, will lose any deuterons attached to electronegative heteroatoms (O, N, S) as the kinetics of their exchange rates are much different than the kinetics of hydrogen exchange of peptide backbone amide hydrogens under quench conditions [79]. Even under quench conditions, deuterons bound to peptide backbone amides will still exchange with hydrogens in solution, so the separation step must be performed as quickly as possible to retain as much of the deuterium label as possible. For an unstructured peptide the exchange half-life under quench conditions is ∼25 minutes [80]. Thus, the exchange rates at the amide groups on the peptide backbone function as a sensitive chain of reporters located along the entire length of a protein where they are involved in the hydrogen bonding networks responsible for protein secondary structure [80].

Hydrogen Exchange Mass Spectrometry

Deuterium is a stable isotope of hydrogen, having an additional neutron and thus each hydrogen exchange with deuterium results in a mass shift of 1.0087 Daltons, an event which can be measured by the mass spectrometer. For an
HDX–MS experiment, an undeuterated protein sample will be subjected to protease digestion. The generated peptides will be separated and tandem MS will be performed to provide amino acid sequence information for the peptides [81]. Because the peptide separations are reproducible, the same peptide will arrive at the mass spectrometer after the same amount of time during each separation, even after it has been subjected to HDX and thus the mass shift due to HDX can be monitored and attributed to the previously identified peptide. A maximally deuterated protein sample must be generated, usually by denaturing the protein with chaotropic agents (compounds which disrupt hydrogen bonds) such as urea or guanidinium chloride and then subjecting the protein to the quench, digestion, separation workflow as normally performed, though a maximally deuterated sample is not necessary for investigations between two states of the same protein, only when absolute exchange information is desired [81, 82]. From the generated mass spectra, deuterium uptake is then determined by calculating the centroid mass of the unlabeled peptide and subtracting this value from the centroid mass of the deuterated peptide. For assessing the performance of the HDX–MS workflow, deuterium back-exchange (BE) is also calculated by:

$$\text{BE} (\%) = \frac{1 - D_{\text{max}}}{N \times D_{\text{frac}}} \times 100$$ (7)

where $D_{\text{max}}$ is the deuterium content of the peptides, $N$ is the number of exchangeable backbone amides of the peptides (calculated as number of amino acids - the number of prolines - 1), and $D_{\text{frac}}$ is the fraction of deuterium in the labeling buffer. Assessing the % BE for standard peptides will ensure system suitability before progressing to measuring precious samples.

Data analysis software options range from commercial suites offered by instrument vendors which require licenses costing upwards of $10,000 to free options. We have used the freely available Mass Spec Studio’s HX–DEAL package for curating our HDX–MS datasets [83].

Though HPLC has been the gold standard in HDX–MS, there has been chatter in recent years that CE would provide a viable alternative [79, 84–86]. If you search google scholar for "capillary electrophoresis hydrogen exchange mass spectrometry" you will find five relevant records besides our own contribution.

There is one example of ambient temperature MCE being used for the separation of labeled, quenched, pepsin digested proteins, this report came out in 2015 and it was indicated they would next cool the device and repeat the experiments, though this has not yet been reported in the literature [87]. This serves as the closest example of using electrophoresis instead of the standard LC based HDX-MS workflow used by HDX labs around the world today. In
one publication of CE HDX–MS they took standard peptides, pre-incubated them in D$_2$O, then performed the separation with fully deuterated BGE and used deuterated SL, using this method to confirm the peptides they had were the peptides they thought they had, based on that all labile protons were exchanged, and this was confirmed by the mass spectrometer [88]. In another two studies, deuterated BGE plugs of different length were injected in the capillary so they filled the distal portion of the capillary and the injected intact proteins would only have contact with a deuterated environment for limited amounts of time at the end of the separation, however the separations were performed using relatively gentle BGE so the proteins, for the most part, maintained their native conformations and were separated by proteoforms while simultaneously being subjected to HDX, and then fragmented from the intact state by ETD, a method known for relatively low deuterium label scrambling during fragmentation [89, 90]. In the fifth report they used deuterated SL to perform HDX at the point of ESI for CE separated small molecule drugs with the result that they observed varying degrees of completeness of exchange for different compounds [91].

Cold Liquid Chromatography
In separating peptides subjected to hydrogen exchange, early on it was found that 0 °C HPLC separations were advantageous to preventing BE and entire HPLC apparatuses were typically submerged in ice baths [92]. 39 years after the first application of 0 °C HPLC for hydrogen exchange, a custom built Peltier cooled HPLC instrument was reported and shortly after offered commercially by Waters Corporation [93]. It is no secret that the separation efficiency of LC is reduced at low temperatures due to the resistance to mass transfer, which is the basis of the reverse-phase LC separations used for HDX workflows [79]. One issue that complicates cold LC separations is the requirement for the instruments to accommodate very high pressures (up to ~20,000 psi [78]. Another problem with cold LC separations is the carryover from previously injected material due to existence of dead volumes in the connections between injection loops, trap columns, protease columns and the reverse-phase column, resulting in the need for rigorous column cleaning procedures to prevent carryover from degrading the spectral data [94]. One study even found that from a consensus list of 169 peptides generated from a ~150 kDa monoclonal antibody, 30% of those peptides exhibited some level of carryover during 0 °C HPLC separations [95]. Despite being the analytical method of choice, 0 °C HPLC definitely suffers from some serious drawbacks.

Cold Capillary Electrophoresis
Most commercial CE instruments provide the ability to control the temperature of the separation capillary by using a liquid cooling system. The main
reason for this is that analyte MT varies by 2 to 3% per °C [20, 21]. The cooling systems are only capable to cool the capillary down to around 10 °C below ambient and thus are not able to support optimal separations in HDX workflows. In the second half of the 1990’s Stacey Ma and Csaba Horvath beefed up the cooling system of their P/ACE 2200 CE instrument to allow for CE separations at temperatures as low as -20 °C, and produced a tripartite body of cold CE research articles, though the work was done with UV detection [96–98]. The only other time subzero separations have been reported comes out of NASA’s Jet Propulsion Laboratory where they were using -20 °C MCE with LIF detection in an ethanol based BGE as the first step in developing a chip-based separation for looking at aliphatic primary amines on Saturn’s moon, Titan [99]. In Paper III we present a laboratory built CE device capable of performing separations down to -8 °C for use in HDX–MS workflows [100].
Aims

The primary aim of the present thesis was the application of cold CE for HDX–MS to be used in subsequent structural proteomics investigations. During the design, fabrication and prototyping of the cooled CE instrument, several other CE instruments were constructed and applied towards additional collaborative efforts, some of which are presented herein.

Paper I

Paper I was focused on the development of a method to easily process some inferior raw materials acquired for the construction of ESI emitters. These emitters were needed for method development towards the objectives of Paper III.

Paper II

Paper II was focused on the development of a CE–MS method for the separation of HNK stereoisomers from pony urine using the PFT approach with CD added to the BGE. There were no reports of this in the literature and previous separations with CE–UV lacked the specificity of MS detection in that UV absorbance of analytes does not provide any additional information of the identity of the analyte and will require analysis of standards to attempt to identify every peak in the electropherogram.

Paper III

The aim of Paper III was the development and validation of a Peltier-cooled CE based separation method to use in an HDX–MS workflow. The wide range of applications accessible by low-cost, lab-built CE instruments has been well documented in the literature and combined with chatter in the HDX–MS community that CE might be a good fit for HDX–MS workflows, the task seemed achievable.
Paper IV

Paper IV was focused on developing a MALDI–MS imaging method for the discovery of potential de novo Parkinson’s Disease (PD) biomarkers in CSF. Tagging phenolic hydroxyl groups and primary/secondary amines was accomplished through the application of FMP–10, a polyphenylated fluoromethylpyridinium reactive matrix [101]. The role of CE–MS in this project was to confirm the identity of an FMP–10 derivatized molecular feature observed at $m/z$ 430.192 which was significantly decreased in PD samples.
Methods

CE–MS

Several variants of a lab-built CE instrument (Papers I–IV) were constructed using a direct current high voltage power supply (HVPS; HPS100-40-0.4, Beijing Excellent Innovate HD Electronics Company, Beijing, China) with detection by a Synapt G2-Si ion mobility- mass spectrometer (Waters Corp, Manchester, United Kingdom). Hyphenation of the CE instruments with the mass spectrometer was accomplished exclusively through the use of a lab-built coaxial sheath flow electrospray ion source similar to a previously described design [23].

Initially (Paper I) the SS hypodermic tubing (270 µm outer diameter (OD), 160 µm ID) used for the electrospray emitters was sourced locally (G. Kinnvall AB, Sparreholm, Sweden). As shown in Panel A, Figure 10, the quality of the unfinished cuts of the tubing resulted in heavy burring and jagged ends of the tubing, making the normal hand polishing with aluminum oxide lapping film more difficult than normal and leading partly to the development of the electrochemical finishing technique presented in Paper I.

Hydrodynamic sample injections were achieved in Papers I, II, IV using an injection platform on which the sample (typically 0.5-2 µL) was deposited, the capillary inlet placed into the sample droplet and then the platform raised 15 cm such that the capillary inlet was 15 cm above the capillary outlet, the platform was then returned to the initial position and the capillary inlet placed into a SS vial containing BGE prior to application of the separation potential.

For enantioselective separations (Paper II), the PFT was used to inject a plug of BGE containing a chiral selector in the proximal length of the capillary. To improve the reproducibility of the plug length, an acrylic manifold was designed in house (Autodesk Fusion, Autodesk, San Rafeal, CA). The manifold could hold vials of the buffers used for capillary conditioning steps between sample injections as well as BGE and BGE containing chiral selector. The manifold was supplied by house nitrogen (g) to provide pneumatic injections of these buffers into the inlet of the separation capillary connected to the manifold with various polyether ether ketone fittings and tubing sleeves. The nitrogen (g) pressure was regulated at the desired value and flow was controlled with a diverting valve (SMC VT307, SMC Corporation, Chiyoda City, Tokyo,
Japan) connected to a timer (Mcr1, Crouzet, Alixan, France) operated by a pushbutton switch (04453, Legrand, Limoges, France). This pneumatic system was modified slightly (see Figure S2 Paper III) with the addition of an additional pressure regulator and a second diverting valve to control capillary conditioning steps and sample injections for Paper III.

Initial experiments (Paper I) used the NanoLockSpray ion source (Waters Corp) with minor modifications for positioning of the electrospray emitter at the MS inlet. Testing of an early prototype of a cold CE instrument revealed incompatible spatial constraints and thermal conditions resulting from the use of the NanoLockSpray source. Thus, an XYZ-linear ball screw gantry stage and control software (FSL40XYZ-L, and AMC4030, Fuyu Technology, Chengdu, China) were acquired and used in subsequent work (Papers II–IV) for positioning of the electrospray emitter at the MS inlet.

Cold CE experiments (Paper III) were performed after the in-house design (Autodesk Fusion, Autodesk, San Rafeal, CA) of an aluminum holder which was machined out of house (DMU 70, DMG MORI, Nagoya, Japan). The aluminum body had a Peltier cooling module (APHC-12704-S, European Thermodynamics Limited, Leicestershire, United Kingdom) affixed to one side to provide thermal regulation of the aluminum holder through the use of a small OEM precision temperature controller with accompanying control software (TEC-1091, and TEC Service software, Meerstetter Engineering, Rubigen, Switzerland). An in house designed aluminum heat spreader was attached to the hot side of the Peltier cooling and cooled by a liquid CPU cooler and associated software (Hydro Series H150i Pro RGB 360 mm, and Corsair iCUE software, Corsair, Fremont, CA).

HDX–MS Data Analysis

Spectra, summed across the peaks of angiotensin II, bradykinin, and methionine enkephalin were subtracted, smoothed, (profile spectra) centered, and then lock mass corrected in MassLynx (Waters Corporation, Manchester, United Kingdom). Centroid masses were manually calculated and BE was determined according to Equation 7. For peptides generated from bovine hemoglobin (Hb), unlabeled peptic digests were separated and MS acquisition was performed in UDMSE mode. Peptide identifications were made with PLGS 3.0.3 (Waters Corporation, Manchester, United Kingdom). Peptide list and waters raw files from the deuterium labeling time points were then curated using Mass Spec Studio’s HX DEAL app [102].
Results and Discussion

Electrochemical etching of stainless steel hypodermic tubing (Paper I)

Austenitic steel tubing commonly used in coaxial sheath flow electrospray sources for CE–MS was etched quickly and cheaply using common laboratory reagents. The benefits of tapered emitter geometries has been previously demonstrated using commercially available tapered tip hypodermic tubing [103]. Such benefits include compatibility with lower SL flow rates, resulting in smaller electrospray droplet sizes and thus enhanced ionization efficiencies and lower limits of detection [103, 104]. Reducing the surface area for anchoring of the electrified meniscus of the Taylor cone also enhances cone jet stability and resulting in more stable droplet trajectories [103]. We showed that SS tapered tip emitters, which are presently not commercially available, can be cheaply and easily fabricated in-house.

Depending on the vendor (Small Parts, Hamilton, Alfa Aesar, etc) pre-cut metal hypodermic tubing may come with some slight burring or imperfections at the cuts. Routine use of metal tubing for ESI will lead to physical degradation of the outlet of the emitter, resulting in deleterious effects to the electrified meniscus at the heart of ESI. Typically imperfections and degradation can be fixed with hand polishing the tubing using diamond or aluminum oxide micropolishing lapping film.

Hand polishing is easier said than done, as the hypodermic tubing ranges from ~150–360 µm in OD, which becomes uncomfortable to pinch between a person’s fingers while gently making figure eights with the terminal face of the tubing on the lapping paper to ensure even polishing. Additionally, the inner lumen of the hypodermic tubing ranges from ~110–180 µm in diameter and in the case of large burs or defects on the cut surface, hand polishing can push metal into the lumen, causing an obstruction that now prevents you from pushing your separation capillary through it during emitter assembly, resulting in time consuming (sometimes unsuccessful) efforts to remove the obstruction. Finally, the wall thickness of the hypodermic tubing is usually between ~50–90 µm thick and excessive manipulation of the tubing inevitably results in catastrophic bending of the fragile metal. What we ended up with is reminiscent of the Ronco Showtime Rotisserie and Barbecue Oven of the late 1990s, where you ”Set it, and forget it”, setting up the electrochemical
reaction, walking away to do whatever other tasks you need to do and then returning later to an etched emitter. The demonstrated benefits of the tapered tip emitters have been documented in the literature though the commercially available parts were no longer longer available at the time of our experiments.

![Figure 10](image.png)

Figure 10. (A) An early batch of locally sourced precut SS hypodermic tubing ordered to use for making ESI emitters. (B) A later batch of precut SS hypodermic tubing ordered from Hamilton Company to use for making ESI emitters.

Enantioselective separation of 6-hydroxynorketamine enantiomers (Paper II)

Ketamine (KET) has recently been the focus of therapeutic treatment of depression in humans. Continued investigations point to the pharmacologically significant activity of the KET metabolite HNK which is produced by the body following KET administration [105, 106]. A CE–MS method for the separation of the four stereoisomers of 6-HNK, (2R,6R-HNK, 2R,6S-HNK, 2S,6S-HNK, and 2S,6R-HNK) was developed. We employed the PFT, which uses a plug of a non-volatile chiral selector injected into the separation capillary with the result that the chiral selector does not exit the capillary outlet and foul the mass spectrometer inlet and ion optics. We have tested both individually and combined in various ratios \( \beta \)- and \( \gamma \)-CDs and have chosen highly sulfated \( \gamma \)-CD for the final method. This is the first report of a CE–MS method for the separation of the 6-HNK stereoisomers. In HPLC fractionated urine from a pony receiving ketamine, we were also able to resolve 2S,4S-HNK
from 2R,4R-HNK, and 2S,4R-HNK from 2R,4S-HNK, and observed multiple peaks corresponding to hydroxyketamine (HK)/methoxynorketamine (MNK). In the absence of chiral selector we also observe KET and its metabolites nor-ketamine, 5,6-dehydronorketamine (DHNK), and dehydroketamine (DHK).

Previously developed CE–UV separations using mixed CDs could perform separation of HNK stereoisomers in under 20 minutes [107]. We attempted to transfer this separation to CE–MS with the assumption that a similar concentration and ratio of CD could deliver similar results. While we ended up with suitable resolution between the HNK stereoisomers, as well additional information regarding DHNK, DHK, and HK/MNK which would be inaccessible to CE–UV without significant extra work, there remain several drawbacks to the CE–MS method. The major disadvantage in our case is that the resolutions achieved required more than 90 minutes of separation time if all four 6-HNK stereoisomers are required. Secondly, because the separations are performed on a laboratory-built instrument requiring manual sample injections, the throughput is not great, and even method development progressed at a snail’s pace. Further investigation of HNKs by CE–MS would absolutely benefit from the use of an autosampler even if the separation length can not be sufficiently shortened.

Cold CE–MS for HDX–MS (Paper III)

The feasibility of cold CE–MS separations in a HDX–MS workflow was demonstrated using a lab-built, Peltier-cooled CE device. This is the first report of the use of cold CE–MS for an in solution labeling HDX workflow. We achieved deuterium BE (20–34% depending on separation conditions), a critical factor for the success of HDX–MS workflows, on par or better than levels achieved by commercial LC based HDX–MS platforms. We also observe plausible deuterium uptake curves across a five orders of magnitude labeling time-course using Hb. Peptide carry-over from previously injected samples requires aggressive and time consuming cleaning steps between injections in LC based HDX–MS workflows, but we were unable to detect a single instance of carry-over during our experiments on protein or peptide standards. Overall, we showed that cold CE appears to be a viable approach for HDX–MS workflows.

There remain many avenues for generating better results from the Peltier-cooled CE device, such as improving the separation efficiency, digestion efficiency, sensitivity, and BE among others. As shown in Figure 11, there are many individual parts to a single HDX–MS experiment, and in our case, in combination with 0 °C CE, none of this has been thoroughly investigated.

One particularly important issue that was not investigated prior to publi-
cation was the tolerance of the system to buffer salts/additives commonly encountered in HDX–MS workflows such as phosphate buffered saline, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES), Tris- (hydroxymethyl) -aminomethane (TRIS), piperazine-N,N′-bis- (2-ethanesulfonic acid), tris(2-carboxyethyl)phosphine, etc. In LC based HDX–MS workflows there is normally a trapping phase where the peptides are loaded onto a trapping column and salts washed away by the aqueous mobile phase prior to elution. There was no such cleanup step in the CE workflow and the ammonium bicarbonate buffer used did not impact the separation or identification of the generated peptides. Following the acceptance of Paper III, some investigation of buffer tolerance was performed in combination with changes to the proteolytic digestion steps and quenching procedure. It was found that a 5 mM concentration of HEPES in the quenched sample would lead to ionization supression as the HEPES plug would migrate out of the separation capillary, drastically reducing the number of identified peptides, while 5 mM TRIS in the quenched sample had no observable effect on ionization.

For Paper III we performed in solution digestion by diluting the Hb 20 fold into a cold, acidic pepsin solution, and vortexing for 5 s before cutting the solution with ice cold acetonitrile (ACN). Without adding ACN, instead of sharp peaks, the peptides come out of the capillary as very broad smears. This single step, the simultaneous quench and digestion offers many options for an improved workflow that will improve both digestion efficiency and sensitivity. In the MCE HDX publication, they used aldehyde coupling to immobilize pepsin onto beads [87]. Immobilizing proteases onto beads or in a monolithic column remain as possibilities but I have come across an equally attractive method where proteases are adsorbed onto porous nylon membranes and can be used in low pressure systems, for example syringe pump driven fluidics providing limited digestion in milliseconds [108]. Initial attempts at incorporating the
modified membranes into our workflow resulted in achieving back-pressures as high as 1500 psi before tubing connector failure. Personal communication between the corresponding author of the modified membranes work later (too late) revealed that despite the reported part number in the manuscript which corresponds to 0.45 µm pore size and 170 µm membrane thickness, the work was actually done with 1.2 µm pore size and 110 µm thick membranes "We almost always use a 1.2 micron pore size with 110 micron thickness. I don’t know that we made any switch...". Outside of any modifications to the digestion procedure, ETD fragmentation was attempted on peptides spanning Hb regions previously reported to be involved in subunit binding in an attempt to localize deuterium uptake at single amino acid resolution. The results of these tandem MS experiments were not meaningfully interpreted despite obtaining quality tandem MS spectra. Due to time and budget constraints, there was no assessment of hydrogen scrambling using standard peptides as recommended [109] for such investigations and this likely contributed to the obtained results.

The starting concentration of Hb was 120 mg/mL, as indicated in the MCE paper, though it was later understood that their injection volume was 600 pL, compared to our ~ 3 nL injection volume. Our 20 fold dilution into deuterated labeling buffer, and second 20 fold dilution into the quench is not standard for HDX workflows, and our recent unfinished work has used a 15 fold dilution into labeling buffer with a 1:1 dilution for the quenching step, allowing us to start with single digit mg/ml protein concentrations. Further tests on limits of detection might be warranted following optimizations of both digestion procedure and BGE optimization.

Referring back to Figure 11 shows that the BGE can be modified in pursuit of both high separation efficiency and low BE. Our choice of BGEs for Paper III both had a pH of ~ 2.2 which is not precisely at the pH where HDX is a minimum, but it did provide for adequate separation of the generated peptides. One potential avenue of investigation would be using BGEs composed of primarily aprotic solvents, limiting the ability for BE with protons in the BGE. Towards this end, during development BGEs of 10–20% dimethylformamide were tested, but resulted in slightly higher BE than with the final BGEs used in the study. In CE there are several techniques to increase the sensitivity by 100–5000 times by adjusting the differences in pH or conductivity between the sample matrix and BGE which result in a focusing or stacking effect [74, 110]. For HDX it seems that adjusting the pH of either the final sample or the BGE is a bad idea, and stacking methods where the entire separation capillary can be loaded with sample are too time consuming. We have recently investigated BGE optimizations looking at simultaneously reducing BE by using pH 2.5 BGE, and improving separation efficiency with modification by buffer additives. In one case, a BGE of 20% HAc and 25 mM AmAc, having a pH of 2.5 provided up to ~ 300,000 theoretical plates for separations of protease
digested Hb, but resulted in \( \sim 80\% \) BE. This just illustrates the kind of challenges facing further method development for CE separations in HDX–MS workflows.

One of the reasons for the success in using small ID (typically 20–75 \( \mu \)m ID) capillaries for separations using high field strengths is that the increased surface area results in efficient heat dissipation through the capillary walls in combination with the reduced band broadening caused by diffusion (with contributions by convective flows induced by Joule heating) within the lumen of these capillaries [7, 111]. In our initial separations we used 40 \( \mu \)m ID separation capillaries, and injected \( \sim 50 \) fmol of protein. After manual curation of the full HDX time-course for Hb and observing quality peptide spectra decay into the background noise over time as increased deuterium uptake broadened the isotopic envelope, it was obvious that an increase in signal intensity would be of benefit for our approach. We obtained some 100 \( \mu \)m ID, 170 \( \mu \)m OD capillary which would allow for \( \sim 6 \) times the amount of sample to be injected in the same plug length as in the 40 \( \mu \)m ID capillaries we initially used. Our assumption was that the Joule heating typically experienced with large bore capillaries would be reduced due to our ability to cool our capillary well below temperatures accessible to commercial CE instruments. The larger OD of the new capillary required the use of 29 gauge hypodermic tubing as the ESI emitter and in combination with the greater contribution of BGE to the eluant, the result was electrospray instability, requiring optimization again of the SL composition and flow rates. Together with time spent on optimization of the LPA coating protocol for the larger ID capillaries and severe current drop experienced during separations on the larger ID capillaries, too many obstacles were encountered with too little time for troubleshooting and pursuit of separations in wide bore capillaries was abandoned.

Two other components on the capillary branch of the fishbone diagram in Figure 11 have been unexplored. There are other capillary coatings available to provide improved peptide separations by eliminating peptide adsorption to the capillary walls. In our case increasing the capillary length would require higher field strengths to sustain the separations, but even at 20 kV, the air gap between the 2 mm push-in fitting (part F, Figure S5, Paper III) on the pod holding the BGE and the body of the CE device would not prevent electrical arcing and separation failure.

Outside of optimizing any of the CE parameters to achieve higher sequence coverage of proteins when using CE for HDX–MS, there are two simple solutions which can be easily provided with today’s MS instruments. Spectral interference due to overlap with chemical background noise or co-migrating peptides decreased the number of usable peptides in our study. The Synapt used in this study achieves a resolution of \( \sim 20,000 \) which is lower than many
other MS instruments available today, so using a higher resolution mass spectrometer would help alleviate some cases of spectral interference. Another common feature on today’s MS instruments (Synapt included) is ion mobility. Much of the method development in our case was conducted on a simple peptide mixture to assess BE, or only looking at half a dozen peptides from Hb using manual calculations to monitor deuterium uptake and MT reproducibility and thus IM was not used for the labeling time-course mostly as an oversight. The zero seconds labeling time-point used for peptide identifications used IM just because this was the DIA workflow which was already established in our laboratory. At a conference I was in a discussion with another scientist about my separations and I alluded to the fact I would next use IM to improve things, I was told not to use that as a crutch and to see how far I can get based on improvements to the CE separations alone.

Identification of norcotinine in human CSF (Paper IV)

A small molecule metabolite detected in a human CSF screening method based on MALDI-MS imaging was identified using CE–MS/MS, resulting in the discovery of a confounding factor in the dataset. The MALDI-MS imaging method involved derivatizing the CSF with FMP–10 to enhance the ionization and detection of molecules with primary/secondary amines and phenolic hydroxyl groups. The method was applied for the detection and identification of metabolic differences between Parkinsonian (de novo PD) and age-matched control patients (n=12 per group). One derivatized molecule detected in the dataset showed statistical significance between the two groups and efforts to confirm the identity with MALDI-MS did not lead to confident structural validation of the metabolite. Using CE–MS/MS, the molecule was tentatively assigned as norcotinine (NCot), a metabolite of nicotine (Nic), though the low abundance (cyan peak, right panel, Figure 12) resulted in low quality MS/MS spectra. Further analysis of the CE–MS data revealed the presence of two additional upstream metabolites from human Nic metabolism, cotinine and hydroxycotinine. The much higher abundance of these two metabolites matched the relative abundance reported from human and we also observed in source decay of the molecules, producing fragments matching previously published triple quadrupole MS multiple reaction monitoring transitions [112–114]. Together these results provided a much higher confidence assignment of the original molecule as being NCot. Finally, comparison of tobacco use recorded in medical records of the human subjects and the samples featuring this peak provided confirmation that this compound was in fact only detected in those who reported tobacco use.
Figure 12. MALDI-MS image inlay showing absence (Sample 1) or presence (Sample 2) of FMP–10 derivatized feature at $m/z$ 430.192 with corresponding CE–MS extracted ion electropherograms revealing additional Nic metabolites.
Conclusions

The work presented in this thesis represents a broad exploration of various capabilities of MS and CE. Several key findings and achievements have been highlighted throughout the text, underscoring the significance of the research conducted. We described a cost-effective method for etching austenitic steel tubing, demonstrating that in-house fabrication of SS tapered tip emitters is a viable alternative when commercial options are unavailable. This development brings greater flexibility and cost-efficiency to electrospray sources, promoting the use of coaxial sheath flow electrospray sources for CE–MS applications. This method makes it easy to access the advantages of tapered emitter geometries, which provide robust electrospray performance and enable lower SL flow rates, resulting in smaller electrospray droplet sizes and lower limits of detection. We introduced a CE–MS method for the separation of the four stereoisomers of 6-HNK, filling a critical gap in analytical capabilities. The use of chiral selectors injected into the separation capillary allows for efficient enantiomer separation, even in complex biological samples. The novel approach not only advances the field but also expands our understanding of the metabolism of ketamine and related compounds. We highlighted the feasibility of cold CE–MS separations in HDX workflows. The use of a lab-designed and built pressure-assisted, Peltier-cooled CE device enables robust HDX–MS analysis with deuterium BE rates comparable to those achieved by commercial HPLC-based platforms. The successful integration of cold CE–MS into HDX workflows opens up new possibilities for studying protein structures and interactions. We used CE–MS to assist with identification of nicotine metabolites in the CSF samples used for a high-throughput MALDI–MS imaging method aimed at diagnosing PD. This contribution revealed the influence of tobacco use on the study’s results and the interpretation of metabolite data in the context of PD. We have identified various challenges and areas for further improvement in CE–MS and HDX–MS workflows. These include issues related to capillary dimensions, buffer compatibility, and BGE optimization, which require continued investigation to enhance sensitivity and efficiency.
Modern life thrives on alternating current (AC) electricity, providing conven-
ience and comfort through things as common as powering a lightbulb. How-
ever, direct current (DC) electricity, less known but stable, is crucial for sensi-
tive electronics, electric vehicles, and medical implants. This thesis explores
capillary electrophoresis (CE), a technique that harnesses high DC voltages
in hollow glass tubes about the thickness of a human hair and filled with a
conductive solution. Since the 1960s, CE has been used to separate and ana-
lyze complex mixtures of DNA, proteins, and many other molecules based on
charge and size.

CE has revolutionized genetic research, drug development, and forensics,
enhancing our understanding of molecular science. Initially, its limitations
stemmed from detecting the separated molecules based on their response to
light as they were moving within the capillaries. Mass spectrometry (MS) has
its beginnings in the early 1900s and is an analytical technique used to mea-
sure a gaseous molecule's mass to charge ratio. In the late 1980s advances
in MS allowed for the coupling of CE with MS (CE–MS) so that the sepa-
rated molecules are able to leave the end of the capillary and go directly into
the mass spectrometer for analysis. Coupling CE with mass spectrometry ex-
panded the utility of CE, as every molecule has mass, but not all interact with
light.

This thesis delves into the diverse applications of CE–MS, investigating
molecules with therapeutic potential for treating depression that don't even
differ in mass, but only in the three dimensional orientation of their chemical
bonds, one of the most difficult challenges to chemists to date. Addi-
tionally, conducting CE separations at 0 degrees Celsius facilitates pinpointing
interactions between proteins and other proteins, or drugs. The future holds
limitless possibilities for CE, contributing to scientific advancements across
various fields.
My supervisor Assoc. Prof. Erik Jansson for accepting me as a Ph.D. student and showing me that I can do science even without a left-handed syringe pump. Thank you for many memorable heart-to-hearts in the gym, on the bike, in the hotel room, Costco, the Mall of America, among other places. Every time you look at the Jordan-shaped dent in your office couch, I hope you are reminded warmly of me and can hear me randomly yelling "Erik!" every few minutes. My co-supervisor, Prof. Per Andrén for filling the lab space with the latest and greatest instrumentation, researchers, and science. Special shoutout to members/collaborators of the Spatial Omics group: Dr. Nilsson, Dr. Shariatgorji, Dr. Fridjonsdottir, Dr. Heijs, Dr. Angerer, Dr. Schembri. It was mostly fun! Dr. Sandbaumhüter for always displaying the highest level of work ethic even amidst the insanity. Many thanks also for letting me join F&D run club. I will be forever grateful to you taking care of all of the urine extractions. You truly were a great coworker. Dr. Kaya for keeping me company in the lab on weekends, helping me to improve my table tennis skills, and always having fresh DEWAIABT data to show me. Also thank you for teaching me the friendly Turkish greeting. I’m sure I will make many friends with it when I visit Türkiye! Dr. Baijnath, dankie broer for the friendship and hospitality. It was a sad and crazy day when you moved from Uppsala, but I’m happy to hear of your meteoric success in ZA. Lotsa love, Uncle F. Assoc. Prof. Hultqvist for introducing me to the amazing world of långfardskridskoákning and letting me tag along with y’all for hundreds of kilometers of the best skating in the world. Thanks to Dr. Ytterberg for the many helpful discussions on sample preparation, measurement, and data analysis and also for valuable feedback on my halftime report. I am a bit sad that I did not discover this asset earlier in my Ph.D. studies. Dr. Double Knee Nika, thank you also for letting me join F&D run club, beer club, hosting many fun dinners, and feeding me too much Jablkovica. Dr. Lodén for being a fun coworker and for letting me take respite at Nordsjön during my cycling trips. Patrik, thanks for making sure that I’ve heard. AFYSOMBASOMB! Oh yeah, oh yeah! Mariya for helping with housing upon our arrival in Uppsala and for all of the delicious desserts. Thanks to the PharmBio gang and adjacents for the fun times: Prof. Söderberg, Prof. Göransson, Quentin, Mingshu, Dr. Slazak, Dr. Rob, Merve, Inga, Andrés, Dr. Rofo, Dr. Kapedev, Dr. Van Der Zwaan, Dr. Al-Takriti, Dr. Sarangi, Dr. Heldin. Dr. Vallianatou, arigatou for the discussions, samples, and snacks! Huge shout out to the guys

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in the BMC workshop. Thanks to Ove Lorentz for the random help. Thanks to Peter Wirsching for help with electronics testing, fabrication, and also letting me use your very nice soldering station. Much respect and thanks to Anders Danielsson for fabricating nearly every component I designed for my research, and also for valuable input into some of the designs and definitely for letting me borrow some big tools when I needed to smash things and cut stuff. Many thanks to Apotekarsocieteten for giving me plenty of financial support so I could present my science at outstanding conferences all over the world.

There are plenty of important people in my life from the times before UU. I must give a lot of credit to those (Prof. Sweedler, Dr. Nemes, Dr. Knolhoff, Dr. Dailey) who showed me the fundamentals of capillary electrophoresis mass spectrometry, giving me the skills needed to conduct all of the research presented in this thesis. Also many thanks to my line managers (Dr. Blumhorst, Qiling), lab directors (Dr. Collison, Dr. Ding) and Principal Scientists (Dr. Nicol, Dr. Hannig) from my industrial research positions for giving me free reign on the CE and CE–MS instruments in the lab. Having access to commercial instrumentation outfitted with software and autosamplers was quite a treat. My habibi, little bird M.D., I’m glad our crazy sojourn with sorcery might finally be over. Much gratitude to my esteemed friend Dr. Neupert for her continued support through letters of recommendation, counseling, scientific discussions over beer, and for being a positive role model scientist. Many thanks to my favorite uber driver, Dr. Bouvier, Ph.D., M.B.A., for assistance with navigating turbulence, tragedy, and transitions in my academic trajectory. I need to thank my undergraduate professors who provided outstanding tutelage and also letters of recommendation at various points in my career: Drs. Wusterbarth, Choudhury, Fierer, Pott, Johnson, and Hencheck. To my sisters: Andrea, Carrie, and Hillary (and one cat who helped proofread my acknowledgements section), thank you for showing me that a somewhat normal life was attainable, despite all of the trauma we endured growing up. To my father, I know you were afraid that I would never leave the house or do anything meaningful with my life. I hope you are finally proud of your baby boy.

My wife Bo for encouraging me to apply for the Ph.D. position even though that came with a massive pay cut and required six relocations before it was all over. Thank you for your unwavering support and making sure I was always fed, hydrated, and sufficiently clad.
References


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A doctoral dissertation from the Faculty of Pharmacy, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy”.)