MICROCHIP INTERNAL STANDARDS

improving quantitative point-of-care
capillary electrophoresis

a dissertation by
Allison C.E. Bidulock

Microchip Internal Standards: Improving Quantitative Point-of- Care Capillary Electrophoresis

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MICROCHIP INTERNAL STANDARDS: IMPROVING QUANTITATIVE POINT-OF-CARE CAPILLARY ELECTROPHORESIS

DISSERTATION

to obtain
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on account of the decision of the graduation committee,
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to my past and those that have touched my life
you taught me **serenity** – strength and courage alone are not enough

to my future and those I have yet to meet
may you be **inspired** – or have a colourful coffee cup coaster

to my present and all those I care for
freely **love, laugh, cry,** and **dance** – occasionally, *all at once*

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CHAPTER 1

introduction

1.1 | Point of Care Diagnostics

Point-of-care tests aim to perform a rapid measurement, either by a physician in close proximity to the patient or by the patient himself, assisting in "bed-side" diagnosis and optimal treatment. While the entire field of point-of-care (POC) diagnostics is very broad (including physical diagnostics methods such as the use of ultrasound devices, stethoscopes, heart rate meters, etc.), here we will use it in a more narrow sense as the determination of (bio)chemical species of the patient.

Considering this more narrow context, the first point-of-care test was developed in 1850, and measured glucose in urine using strips of sheep's wool containing stannous chloride.^{1,2} A number of paper-based tests were introduced in the early 1900s by George Oliver³, followed by many other test strips marketed in the 1950s. The field saw a step in sophistication when lateral flow devices appeared in the 1980s, which applied immunoreactions for highly sensitive measurements such as that of the hCG hormone in the pregnancy test.⁴ Quantification also became also possible in the 1980s, firstly with a test for determining the glucose concentration in the blood of diabetes patients.⁵ Then the I-Stat analyzer appeared in the 1990s, a handheld reader with a series of cartridges that could be applied for different analytes.⁶ Presently the I-Stat has acquired a prominent role in point-of-care analysis in the clinical practice.

Since the 1990s, the highly sophisticated micromachining methods that had been developed by the electronics industry (mainly silicon) and the polymer industry (e.g. micromolding as used for CDs) started being used to produce chemical analysis systems. These systems were termed μ TAS (micro Total Analysis Systems) or "Lab-on-a-Chip" devices.⁷ They represented the next step in a continuous technological evolution that generated increasingly sophisticated devices. Quantitative measurements now became more the rule than the exception, a new range of analytes could be targeted, and POC diagnostics could be used in the development of personalized medicine.

The knowledge that was acquired from the 1980s onward is, at present, somewhat injected back into the original paper-based diagnostics via the field of paper-based microfluidics or "Lab-on-Paper", which has become quite popular in the last decade.^{1,2} The lab-on-paper diagnostics aim to drastically reduce the production costs of the device by using the cheaper paper material and machining processes, instead of relying on the more expensive glass/silicon materials and micromachining methods.

The Lab-on-a-Chip technology has also enabled the use of capillary electrophoresis for point-of-care analysis, by micromachining the capillary electrophoresis capillary in a microchip. This thesis reports on the investigation of methods to use an internal standard in point-of-care microchip capillary electrophoresis to improve analyte quantification.

1.2 | Capillary Electrophoresis

Capillary electrophoresis is a method for the separation of ions. In 1958, Arne Tiselius received the Nobel prize in Chemistry for his invention of capillary electrophoresis.³ To perform electrophoresis, an electrical field is applied in a solution by applying a voltage difference between two electrodes (Figure 1.2-1). As a consequence, a force is exerted on all ions present: positive ions will move towards the negative electrode and negative ions towards the positive electrode. In the same field, dissimilar ionic species will move with different velocities because either the force that the field exerts on them differs (when they have different charge), or their friction with the solution differs (when they have different size). We can thus separate ionic species in the applied electrical field.

To perform a stable measurement, it is good to perform the separation in a long channel or tube serving as a conduit for ionic movement. First, a small plug of sample is injected into one side of this channel by temporarily applying pressure or an electrical field. This is then followed by the application of the separation field, after which the ions will move with different velocity through the channel. When a detector is placed somewhere along the channel axis, we will see the different analytes pass at different times.

Figure 1.2-1. Schematic of a separation by capillary electrophoresis. A plug of two substances with a different mobility in an applied electrical field is separated by the application of a field along the axis of a channel.

Popular detection methods thereby are UV adsorption, fluorescence, and conductivity detection. The analyte can be quantified by the proportionality between the detector peak height or area and the amount of analyte, while its arrival time is indicative of its identity. Unfortunately, all kinds of variations in the measurement circumstances in CE will lead to imprecision in the analyte determination. The addition of internal standards can strongly improve the analysis, as was reported in a number of papers in the 1990s.^{8,9,10}

1.3 | Microchip Capillary Electrophoresis

Since the seminal work of Harrison and Manz in 1991 that inaugurated the μ TAS era, much literature has appeared on the use of microchips for capillary electrophoresis.¹¹ Due to the modest instrumental needs (high voltage sources can be easily downscaled), microchip CE lends itself well to POC or Point-of-Use (POU) determination. For the later developments in this area including the application to point-of-care diagnostics, recent reviews can be consulted.^{12,13,14} Only a few investigations have appeared investigating the reproducibility of repeated measurements using a single chip. Importantly, regarding the subject of this thesis, no investigations have systematically investigated the chip-to-chip reproducibility in microchip CE, or the addition of an internal standard.

1.3.1 | application to point-of-care diagnostics

Microchip capillary electrophoresis (MCE) for point-of-care diagnosis was first developed in the van den Berg group and reported in a series of papers by Vrouwe et al.¹⁵ Vrouwe used MCE with conductivity detection and applied the method to the detection of lithium in human serum, a drug used in the treatment of manic depression (bipolar disorder). The work of Vrouwe was taken up by start-up company Medimate, which developed a POC device for the measurement of lithium. The device consists of a handheld reader (Medireader) and a disposable cartridge containing a prefilled microchip (Figure 1.3-1).

Figure 1.3-1. The Medimate Medireader, a point-of-care diagnostics device based on microchip capillary electrophoresis with conductivity detector. The plastic cartridge contains a microchip which after application of a drop of blood obtained with a fingerstick is inserted in the handheld reader.

The company published several papers from 2010-2015 reporting on the performance of this system, showing continuous improvement and application to other analytes than lithium.^{16,17,18} A second spinoff company, Blue4Green, developed the device for the veterinary market. In 2015, Medimate became a daughter company of CE-Mate, developing the technology for the general point-of-use market.

Though the Medimate Medireader has good accuracy and precision, there is certainly still room for improvement. One of the largest problems to be tackled is the chip-to-chip variation. The microchips are produced in the cleanroom by wet etching of channels in a glass wafer, deposition of metal electrodes to measure the conductivity and to apply the high voltages, powder blasting of reservoirs and openings, and high-temperature fusion bonding to a second chip to close the device. Though the production procedures have been standardized, some chip-to-chip variation is bound to remain. Other sources of variation in the Medireader output will be variations in magnitude and duration of the applied electrical potentials. Finally, variations in the environment (humidity, temperature) and the operator will occur between measurements. All these factors will

cause variation in the final outcome of the measurement, leading to decreased precision and accuracy. Such variations must certainly be reduced as much as possible in a clinical application, since therapy decisions that carry considerable risk can depend on it.

To reduce the residual error, it is common practice in analytical chemistry to add an internal standard to the sample. An internal standard is a substance similar to the analyte but not present in the sample, which is added to the sample in a known concentration. The internal standard then undergoes the same analysis steps under the same circumstances as the analyte, and the internal standard peak can be used to correct the analyte peak leading to improved quantification. In a laboratory setting, an internal standard can simply be added to the sample by a skilled operator with a pipette and subsequent mixing. In a point-of-care setting where the measurement is performed by an unskilled operator this is impossible and another method must be sought. Complicated automated procedures to add the internal standard will easily create new sources of variance, and a simple method is thus strongly preferred. The work reported in this thesis aims at developing a simple and robust method for internal standard addition in microchip capillary electrophoresis.

1.4 | Scope of Thesis

This thesis reports on the investigation of methods to use an internal standard to improve quantification in point-of-care microchip capillary electrophoresis. In chapter 2, the reader will be introduced in the main principles of CE, the different processes occurring during a CE analysis, how these affect the analyte quantification, and how the quantification can be improved by different procedures. Chapter 3 investigates what the main sources of error in chip-to-chip precision are and how internal standards account for these errors. In chapter 4, a new approach is presented, whereby the same internal standards added to the sample in chapter 3 are instead added to the background electrolyte. Chapter 5 then builds upon this new approach by investigating the chip-to-chip precision with a relatively controlled sample matrix similar to blood. Chapter 6 finally presents a summary and suggestions for further research. An appendix finally is added to provide details of the setup and measurement process.

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CHAPTER 2

theory

2.1 | Introduction

In this chapter, an overview is given of the main aspects of capillary electrophoresis that are relevant to the work presented in this thesis; consecutively: the separation mechanism, the requirements of the background electrolyte, mechanisms of peak dispersion and how to prevent these, electroosmotic flow, detection sensitivity, and the use of an internal standard will be treated.

2.2 | Separating Ionic Species

In capillary electrophoresis, different ionic species are separated in a capillary or a channel by applying an electrical field along the channel axis. Dispersed ionic molecules in an aqueous environment will become mobile when an electric field is applied across the solution. If the electrical field E is spatially homogeneous, the ions will move towards the opposite pole with a velocity:

$$v = \mu_E E \quad \text{Eq. 2.2-1}$$

where μ_E is the electrophoretic mobility of the charged particle and E is the external electric field. μ_E is dependent on the electrostatic force driving the ion's movement and the counteractive resistive viscous force of the fluidic medium on the molecule. During

electrophoresis, a steady-state will be achieved where the two opposing forces will be equal. This defines the electrophoretic mobility as:

$$\mu_E = \frac{zq}{6\pi\eta r} \quad \text{Eq. 2.2-2}$$

where z is the ionic charge number, q is the unit charge ($1.6 \cdot 10^{-19}$ C), r is the ion's Stokes' radius, and η is the viscosity of the fluidic medium. The mobility of a charged particle under an applied electric field is thus dependent on its charge and size. Different ionic species have different electrophoretic mobilities and hence different migration velocities in an applied electric field, allowing ions to be separated into specific regions or zones based on their charge and size.

It should be noted that in reality, a species' ionic charge, radius and thus electrophoretic mobility can vary within a specific experimentation set due to temperature, pH and ionic strength. In practice, these effects are often considered to have similar influence on all ions within the electrophoretic system, and the *relative mobilities* between all species are generally considered constant.

2.3 | The Background Electrolyte in Electrophoresis

The background electrolyte's (BGE) primary role in electrophoresis is to provide an electrically conductive medium in which analytes of interest will migrate past a detector selectively and in reasonable time, with minimal dispersion. As its physiochemical and electrical properties influence many critical aspects of the electrophoretic process, the BGE's role in the performance of quantitative capillary electrophoresis is very complex.

Principles of the BGE and non-idealities will first be described to provide a basis for understanding the purpose and influences of the BGE in capillary electrophoresis. This foundation will then be used to identify sources of variability in capillary electrophoresis which pertain specifically to the BGE and compile a list of factors to consider when choosing the BGE. Finally, buffers commonly used in the literature for the quantitative detection of inorganic ions will be reviewed.

2.3.1 | electrically conductive medium

In classical capillary electrophoresis, a column is filled with the background electrolyte to allow the migration and/or transport of analytes when a voltage is applied across the capillary. The uniform cross-section of the capillary results in a constant current density, J , so that when it is filled solely with a solution of conductivity, κ ($\Omega^{-1}\text{m}^{-1}$), a constant

electrical field strength, E , will be applied to the ionic constituents within the capillary via the modified Ohm's law:

$$J = \kappa E \quad \text{Eq. 2.3-1}$$

As can be seen above, an electrically conductive medium (i.e. $\kappa \neq 0$) is required to facilitate the current flow.

To be electrically conductive and at the same time preserve electroneutrality, the BGE must contain an equal amount of cationic and anionic charge, and therefore at least one cationic and one anionic species. The BGE ion that shares like charge with the analyte(s) is called the co-ion, while the oppositely charged BGE ion is referred to as the counter-ion. The conductivity of the BGE is determined by the concentrations, c_i , and absolute mobilities, $|\mu_i|$, of all ionic constituents present in the solution given by:

$$\kappa = F \sum_i^n z_i \mu_i c_i \quad \text{Eq. 2.3-2}$$

where z_i is the charge number and F is Faraday's constant.

2.3.2 | capillary zones and boundaries

The capillary will not only ever be filled with the BGE alone. Analytes will be introduced into the system and separated based on their mobilities; this is the principle of capillary electrophoresis. At any given time, the capillary can be said to have j "zones": multiple lengths of unique sets of ionic constituents, i_j . These zones will then have different specific conductivities, κ_j (equation 2.3-2), and furthermore, will experience different electrical field strengths, E_j (equation 2.3-1), due to a constant current density that needs to be running through the system. The interfaces of these unique zones are referred to as "moving" boundaries.

The Kohlrausch Function, or KRF, is a conservation function in electrochemistry that states while the concentrations of any constituents of the electrolyte may vary with position, x , and time, t , there exists a numerical value (in $\text{mol} \cdot \text{V} \cdot \text{s} / \text{m}^5$) at each co-ordinate in the system that is locally invariant with time (assuming migration as the system's only mass transport mode). This value is defined by¹:

$$\text{KRF}(x) = \sum_i^n \frac{|z_i| \cdot c_i(x, t)}{|\mu_i|} \quad \text{Eq. 2.3-3}$$

Supposing an electrokinetic injection set-up (Figure 2.3-1, left), a capillary length is filled solely with BGE at $t_{inj,0}$ and the KRF is the same value at every position along the capillary, determined by the composition and concentration of the BGE. At $t_{inj}>0$, the electric field is applied and ions begin to migrate from the significantly large sample volume into the much smaller capillary (electrophoresis begins). However, an invisible and unmoving ("stationary") boundary exists at the interface between the sample and the BGE/capillary. As the analyte ions cross this boundary, they replace the BGE co-ions and must either concentrate ("stack") or dilute to conserve the KRF.

In a separation set-up (Figure 2.3-1, right), at $t_{sep,0}$ the electrokinetically- or hydrodynamically-injected analytes are located within a specific region of the capillary, surrounded by the BGE. Here, two stationary boundaries are present at $t_{sep}>0$: one at each interface between the initial sample region and the BGE. However, the analyte will only cross the stationary boundary that is on its migration path and will also concentrate or dilute to conserve the KRF in that region.

Figure 2.3-1. Depiction of zone boundaries formed by the migration of a single analyte when an electric field is applied across the capillary at $t > 0$. KRF_a and KRF_b are two independent KRF values, κ_{A1} is the initial analyte zone conductivity and κ_{A2} is the concentrated/diluted analyte zone conductivity.

While conservation of the KRF leads to concentration or dilution of a migrating analyte region across a stationary boundary, in both electrokinetic injection and separation, the determining KRF value is decided at t_0 by the background electrolyte. Consider a system with two unique, monovalent species: BGE co-ion (i) and analyte (j). Ignoring the BGE counter-ion for simplicity, as it is present in both zones to preserve electroneutrality, the concentration of the new analyte region will be:

$$\sum_i^n \frac{|z_i| \cdot c_i(x_i, t = 0)}{|\mu_i|} = \sum_j^n \frac{|z_j| \cdot c_j(x_i, t > 0)}{|\mu_j|}$$

Eq. 2.3-4

$$\rightarrow c_j(x_i, t > 0) = \frac{|\mu_j| \cdot c_i}{|\mu_i|}$$

Thus, the *absolute* concentration of an electrokinetically injected analyte of theoretically infinite amount is determined by the BGE and not its initial concentration in the sample. If only a finite injected amount of analyte is present (as in a typical separation), the width of the new analyte zone will be determined by the amount of analyte available to reach the concentration demanded by the conserved KRF.

2.3.3 | capillary zone electrophoresis & moving boundary electrophoresis

Now consider that a small solution volume (plug) is present in the column that contains an analyte set that consists of a number of monovalent ionic species (*a*, *b*, *c*) with different mobilities ($\mu_a > \mu_b > \mu_c$). The analyte ion with the fastest mobility (*a*) will cross the stationary boundary with the highest velocity, *b* slower and *c* still slower. The analyte set will thus split into individual zones separated by BGE. This process is graphically represented in the bottom of Figure 2.3-2 and is also called capillary zone electrophoresis. Throughout this thesis, capillary zone electrophoresis is used for analyte separation.

If a theoretically infinite amount of the analyte set is present, as in an electrokinetic injection from a very large sample reservoir, a different effect will be observed. Again, the analyte ion with the highest mobility (*a*) will cross the stationary boundary the fastest, *b* slower and *c* still slower. However, in this case, individual zones of each analyte will not be formed since more and more analyte will continue to cross the stationary boundary. With our analyte set of three ions (*a*, *b*, *c*), three specific analyte zones will then form: first *a*; then *a* + *b*; and, finally *a* + *b* + *c*. This process is depicted in the top of Figure 2.3-2 and can also be referred to as "moving boundary electrophoresis". Throughout this thesis this process is used for analyte injection.

The absolute concentrations in these zones will be determined by the KRF, as in the single analyte system. The *relative* concentrations of *a* : *b* in the second zone and *a* : *b* : *c* in the final zone, however, are determined by the sample concentrations and constituents.²

Figure 2.3-2. Representation of the zones formed when an electric field is applied to a finite (bottom) and infinite (top) amount of an analyte ionic constituent set (a, b, c). The process at the top is called moving boundary electrophoresis and at the bottom capillary zone electrophoresis.

2.3.4 | buffering system

The pH of the background electrolyte directly influences the effective mobilities of many species that undergo protonation equilibria— sample analytes and BGE constituents alike – which can have numerous effects on the electrophoresis process. For reproducible and quantitative detection in capillary electrophoresis, the pH is critical and its regulation is the second primary task of the BGE. For this reason, the terms "background electrolyte" and "buffer" are often used interchangeably in the literature.

Buffering

For the weak acid HA we have the equilibrium

The acid dissociation constant, K_a , is a measure of the strength or affinity for protons of the acid, and is written as the quotient of the equilibrium constituent's concentrations:

$$K_{a,A} = \frac{[\text{H}^+][\text{A}^-]}{[\text{HA}]}, \quad \text{Eq. 2.3-6}$$

Taking the logarithm of $K_{a,A}$, we derive the Henderson-Hasselbalch equation:

$$\log K_{a,A} = \log[H^+] + \log \frac{[A^-]}{[HA]} \rightarrow \text{pH} = \text{p}K_{a,A} + \log \frac{[A^-]}{[HA]} \quad \text{Eq. 2.3-7}$$

where the $\text{p}K_a$ is the negative logarithm of the acid dissociation constant. This equation illustrates two properties of an acid: a) the extent of dissociation of a weak acid depends on the pH of the solution and the species' $\text{p}K_a$; and, b) the pH at which the weak acid is 50% dissociated is equal to the $\text{p}K_a$ of the acid (i.e. $[A^-] = [HA]$ and $\log 1 = 0$).

We can further relate the concentration of the associated/dissociated species to the pH and the total concentration of the weak acid solution with (where c_A is the total concentration of acid):

$$[A^-] = \frac{c_A}{10^{\text{p}K_a - \text{pH}} + 1} \quad \& \quad [HA] = \frac{c_A}{10^{\text{pH} - \text{p}K_a} + 1} \quad \text{Eq. 2.3-8}$$

To illustrate these relations, the dissociation of 1M acetic acid ($\text{p}K_a = 4.756$) as a function of pH is plotted in Figure 2.3-3.

Figure 2.3-3. Illustration of the buffering capacity of an ideal solution of 1M acetic acid.

General buffer solutions

The simplest buffer solution is a solution of a single weak acid or base. Much like a capacitor opposing voltage changes in electronics, the buffer opposes pH changes and its strength to do so is called the buffer capacity, β . We can illustrate this mathematically by taking the derivative of equation 2.3-8, which represents the change in dissociation of X needed to achieve a small change in pH:

$$\beta = \frac{\partial c_Y}{\partial [A^-]} \frac{\partial [A^-]}{\partial \text{pH}} = \frac{\partial c_Y}{\partial [A^-]} \ln 10 \frac{c_A \cdot 10^{pK_a - \text{pH}}}{(10^{pK_a - \text{pH}} + 1)^2} \quad \text{Eq. 2.3-9}$$

where c_Y is the concentration of added strong acid/base species Y in the solution, and $\partial c_Y / \partial [A^-]$ represents the amount of strong acid or base needed to change the dissociation of A. If the added strong acid/base is monovalent, then $\partial c_Y / \partial [A^-] = 1$ as one molecule of Y will donate/accept one hydronium ion; if it is bivalent, then $\partial c_Y / \partial [A^-] = 0.5$; etc.

The buffer capacity as a derivative of the weak acid dissociation is illustrated for 1M of acetic acid in Figure 2.3-3. Monovalent acids/bases with different pK_a will have the same curve, with the maximum buffer capacity at $\text{pH} = pK_a$. The buffering capacity can only be increased by increasing the concentration, c_X ; however, at large concentrations this becomes impractical. Thus, more complex buffer systems are formed by mixing multiple weak acids and/or bases, and/or using polyprotic weak electrolytes with multiple acid-base conjugates (e.g. H_2A , HA^- , A^{2-}) so multiple pK_a values will exist, and equation 2.3-9 becomes more complex.

To prepare a buffer solution, a weak acid/base is therefore titrated with either its conjugate salt (e.g. NaX_b) or a strong acid/base (e.g. HCl) to bring the buffer solution to the desired pH. In a simple buffer solution, this pH should be close to the pK_a for optimal buffering capacity; in more complex buffer systems, unfortunately no simple rule exists³.

2.3.5 | system zones

In the previous section, we discussed different specific zones within the capillary, made up of the BGE and various analyte constituents and concentrations. In some BGE systems, however, additional zones also appear with specific mobilities that can deform analyte zones migrating within their vicinity. No analytes are contained within these zones; they are a function of the BGE system. Thus, they are often referred to as "system zones", although they can also be referred to as "eigenzones"⁴.

For a BGE consisting of n co-ion constituents, there will be $n-1$ system zones in the capillary. In a BGE system containing two co-ions, the resultant system peak will have an

electrophoretic mobility between the two co-ions, and will reside closer to the co-ion with the lowest concentration.⁵ In a BGE system of co-ions *A* and *C*, and counter-ion *B*, the system peak mobility λ_c due to the addition of co-ion *C* to the BGE, can be derived from equation 40 of Štědrý⁶ as:

$$\lambda_c = \frac{2c_A c_B (\mu_A + \mu_B) \mu_C + c_A c_C (\mu_A + \mu_C) \mu_B + c_B c_C (\mu_B - \mu_C) \mu_A}{(\mu_A c_A + \mu_B c_B + \mu_C c_C)(c_A + c_B)} \quad \text{Eq. 2.3-10}$$

where *c* is concentration and μ is mobility. This equation assumes that H^+ and OH^- can be neglected (the pH is relatively neutral), buffering ions *A* and *B* are half-ionised, and co-ion *C* is entirely ionized.

Multiple co-ion systems are not the only BGEs with system zones; very high or low pH systems, and BGEs with a multivalent weak acid(s) or base(s) at a pH near the pK_a value of an ion-ion conjugate pair, will also form system zones. While the mobility of the system peak is relatively simple to estimate, the amplitude of system peaks is more difficult due to their inherent dependence on the sample matrix⁴. Mathematical models and/or simulation programs, such as Simul 5⁷, are often used to predict the mobilities and amplitudes of system peaks.

2.3.6 | impurities

BGEs are mixed with deionized (in some applications, also autoclaved) water and commercial, standard materials using thoroughly cleaned equipment with utmost care so that no impurities are introduced into the buffer. Aside from noting that this is never the case in reality, there is one impurity available in copious amounts and unavoidably in contact with the solution at all times: carbon dioxide.

Deionized water in a chemical laboratory includes at least the following reactions³:

Thus, in practice, the pH of laboratory water is never 7 and the pH of a solution must always be measured after dilution.

The dissolution of carbon dioxide also changes the solution's ionic strength (and thus, the pK_a and ionized mobilities). Below pH 5.5, $CO_{2(aq)}$ is dominant and ionized conjugates are

minimal; however, above pH 5.5, the contributions to ionic strength can be significant and on the order of ~10mM.³

2.4 | Quality of Separation: Efficiency and Resolution

A description of the performance of capillary electrophoresis is required to compare protocols using one system and between separation systems. As with any analytical chemistry process, the performance can be broken in many separate and distinctive components, including the system's efficiency, resolution, accuracy and precision.

The efficiency of a separation system directly determines its ability to resolve analytes from one another and the remaining components present in the sample matrix, and thus the resolution of a system is often used to describe its performance. In capillary electrophoresis, the resolution is decided by the location of the peak maximums (the peak migration or apparent mobility, discussed previously) and the peak width or dispersion. For example, two analytes with similar apparent mobilities may be resolvable if the peaks are narrow or may appear as one large peak if the peaks are wide. For this reason, it is important to understand what factors affect the peak dispersion.

2.4.1 | the ideal analyte peak shape

At initial separation conditions, an infinitesimally thin boundary will exist between the analyte zone and the surrounding buffer. This boundary will subsequently deteriorate during separation due to diffusion across the high concentration gradient. Application of Fick's second law of diffusion describes the longitudinal dispersion of ions across the analyte zone as a Gaussian curve⁸, with peak maximum dependent on the initial concentration of analyte. The width of the zone is dependent on both the time t elapsed between the initial creation of the infinitesimally thin boundary and the analyte's arrival at the detector, and diffusion co-efficient⁹, D :

$$D = \frac{\mu_E kT}{zq} \quad \text{Eq. 2.4-1}$$

Knowing from probability theory that the width of the Gaussian analyte band is characterized by its standard deviation σ ¹⁰ we obtain the analyte band width as

$$\sigma = \sqrt{(2Dt)} \quad \text{Eq. 2.4-2}$$

2.4.2 | plate theory of separation

Like its chromatographic separation system predecessors, the efficiency of capillary electrophoresis is sometimes expressed using the theoretical plate height.

Plate theory has two assumptions¹¹: a) the channel can be divided into a large number of 'theoretical plates' containing the stationary phase and the mobile phase; and, b) as the analyte passes through each plate, equilibrium between the stationary and mobile phases is reached instantaneously. The plate must therefore be of sufficient length to allow equilibrium to be established; the thinner the plate, the faster the equilibrium. The total number of plates of a column is used to define the separation efficiency of the system¹², and written:

$$N = \frac{L^2}{\sigma^2} \quad \text{Eq. 2.4-3}$$

where N is the plate number, L is the length of the channel, and σ^2 is the variance or width of each plate.

When diffusion is the only cause of peak dispersion (as assumed with the ideal peak shape), the standard deviation σ of the Gaussian curve is a function of the diffusion coefficient and time as described in the previous section. Substituting into the above equation $\sigma^2 = 2Dt$ and $t = L^2/\mu_E V$ (assuming $\mu_{\text{eff}} = \mu_E$), the plate number can be written as follows, where V is the applied voltage¹³:

$$N = \frac{\mu_E V}{2D} \approx 20|z|V \quad \text{Eq. 2.4-4}$$

This demonstrates that when diffusion is the only source of peak broadening in the system, the efficiency is mainly dependent on the time it takes the analyte to reach the detector. A higher applied voltage will cause the analyte to move faster through the channel, reducing the time and thus increasing the efficiency. While in a well-controlled experiment diffusion is the major contributor to peak broadening, other phenomena contribute as well and it is important to understand the other effects on the peak shape and its variability.

2.4.3 | other sources of peak dispersion

Extra-column effects

The effects of the channel size, shape and detection system on peak dispersion are collectively termed extra-column effects. Plate theory assumes the sample plug at the

start of separation and the detection window to be infinitely small⁹, both of which are very finite in reality. It also assumes the channel to be straight along its entire length, which is not always possible on microfluidic chips containing other integrated functionalities.

To prevent over-loading of the sample and significant extra-column effects, the rule of thumb in capillary electrophoresis is that the injection plug volume must be 1% of the entire separation channel length, at most⁸. However, it is important to note that injection volumes are highly variable in capillary electrophoresis and some argue that even this is too large to eliminate injection effects⁹.

Adsorption and electro-osmotic flow

As illustrated in Eq.12, the length of time the analyte spends in the separation channel mainly determines the efficiency of the system. Thus, the channel walls are usually coated to prevent adsorption of the analyte, which can considerably slow the peak's progression through the channel or even cause the adsorbed analyte to never reach the detector. Adsorption can also lead to peak tailing.

In most instances, these coatings are used to limit EOF as well as adsorption. Any analyte with electrophoretic mobility in the opposite direction of the EOF will progress slower through the channel, increasing diffusion and peak width. Furthermore, depending on the channel wall material and/or the fabrication process, differences in the zeta potential or charge on the capillary walls can exist along its length, resulting in nonhomogeneous EOF and laminar flow.

Electrodispersion

When an analyte is not encountering stationary boundaries, its migration through the capillary is determined by electrophoretic processes. Practically, this means that broadening of any newly concentrated analyte zones begins. Several effects define the form of moving boundaries, and thus, the width and shape of the detected analyte peak. One of the more significant effects, which pertains specifically to the BGE, is interchangeably called the electrodispersive effect or electromigration dispersion (EMD).

The KRF is locally invariant with time, but the conductivity is not. The analyte zone will have one conductivity within the KRF region of its initial starting region, and another conductivity on the other side of the stationary boundary where it is concentrated/diluted, as seen by the c_i term in equation 2.3-2; (refer back to Figure 2.3-1).

Assuming an analyte zone, j , has completely crossed the stationary boundary, it can experience one electric field (say, E_j) from equation 2.3-1 and the BGE can experience another (say, E_{BGE}). If an analyte ion migrates across the leading moving boundary into the background electrolyte zone and $E_{BGE} < E_j$, its velocity will slow (as the electric force exerted on it is lower) and it will be re-captured by the faster moving analyte zone. This leads to a stabilized, "self-sharpening" boundary. However, if $E_{BGE} > E_j$, the analyte ion's velocity will increase and it will move further and further ahead of its zone with time, resulting in a non-stabilized, broad boundary. This is conceptualized graphically in Figure 2.4-1.

Figure 2.4-1. Illustration of the formation of stabilized/self-sharpening and non-stabilized/broad moving boundaries during separation due to different relative electric fields.

Mikkers et al.¹⁴ showed that the analyte zone's electric field, E_j , is directly related to the relative mobilities of the BGE co-ion and the analyte ion via the KRF. This is not very surprising when, considering a zone with an ionic constituent set, i , the relationship between the KRF and conductivity of this zone, κ_i , can be written at t_0 as:

$$\frac{\kappa_i(x_i, t_0)}{\text{KRF}_i(x_i)} = \frac{F \sum_i^n |z_i| \cdot \mu_i \cdot c_i(x_i, t_0)}{\sum_i^n \frac{|z_i| \cdot c_i(x_i, t_0)}{\mu_i}} = F \sum_i^n \mu_i^2 \quad \text{Eq. 2.4-6}$$

Defining the relative mobility as $r_j = \mu_j / \mu_{co-ion}$, the leading boundary as the first moving boundary in the direction of migration and the trailing boundary as the second moving boundary, then the analyte zone will experience electrodispersion as described in Table 2.4-1. If $r_j = 1$, then the electric fields will also be equal and neither moving boundary will be sharp nor broad; the analyte will only be concentrated or diluted across the KRF stationary boundary.

Table 2.4-1. Electrodispersive effects on the analyte zone due to analyte-BGE relative mobility, summarized.

		Tailing Boundary	Leading Boundary
$r_j < 1$,	$E_{BGE} < E_j$	broad	sharp
$r_j > 1$,	$E_{BGE} > E_j$	sharp	broad

For optimal selectivity or resolution, the analyte zone should be as thin and concentrated as possible. Thus, to limit broadening from electrodispersive effects, the BGE co-ion is often chosen to have an effective mobility closely matching the analyte(s) of interest.

Thus, among other properties discussed later, the conductivity of the analyte's surrounding environment must be deliberately chosen and strictly controlled to achieve efficient and reproducible separations. This is the first primary task of the BGE.

Joule heating

When an electric current carried by ions passes through a solution, friction between the moving ions and the solvent molecules results in a portion of the applied electrical energy being transferred to the solution as thermal energy. This process is called Joule heating. Depending on how much energy is transferred and the effectiveness of the solution and its environment in dissipating this additional heat to its surrounding environment, the solution temperature can increase by negligible or significant amounts. In capillary electrophoresis, the amount of Joule heat generated is determined by the applied electric field and the BGE's electrical and thermal properties, while the efficiency of heat dissipation is determined by the capillary dimensions and thermal mass as well as the environment of the capillary (which can e.g. be cooled).

Absolute increases in the system temperature are generally not considered to be detrimental to the separation efficiency of capillary electrophoresis¹⁵, and can even be advantageous in some applications such as protein detection⁸. The main negative consequence of Joule heating is the radial temperature gradients across the capillary cross-sectional area due to the finite rate of heat transfer and higher thermal dissipation at the capillary walls. These temperature gradients can have adverse effects on the efficiency via three properties of the BGE: viscosity, density and pH. Since in the system used in this thesis the dissipated energy can be neglected due to the small currents, large surface-area-to-volume ratio, and the large thermal mass of the glass chip, we will not further consider Joule heating here.

2.4.4 | determining resolution

Using the full-width half maximum (FWHM) to describe the width of the peak, the number of theoretical plates can be expressed as⁸:

$$N = \frac{L^2}{\sigma^2} = 5.54(L/w_{FWHM})^2 \quad \text{Eq. 2.4-7}$$

where N is the plate number, L is the length of the channel, σ is the standard deviation, and w_{FWHM} is the width of the peak at half maximum.

2.4.5 | electro-osmotic flow

One major influence on an ionic species' electrophoretic migration velocity specific to capillary electrophoresis is the phenomenon of electro-osmotic flow (EOF). Acidic silanol groups on the walls of many capillary materials acquire a negative charge by deprotonation when in contact with the background electrolyte. Cations then accumulate close to the surface of the wall to maintain electro-neutrality, forming an electric double layer. When an axial electric field is applied for electrophoresis, these cations experience a force directed at to the negative electrode and transfer their momentum to the solution molecules. This causes the electrolyte to flow along the capillary, with a mobility determined by the charge on the capillary (as quantified by the potential at the so-called plane of shear where the solution shears along the wall, ζ), the electrolyte viscosity (η) and the dielectric constant of the electrolyte (ϵ) using¹⁶:

$$\mu_{EOF} = -\frac{\epsilon\zeta}{\eta} \quad \text{Eq. 2.4-8}$$

This also affects the migration velocity of the analytes, positively or negatively depending on the ion's charge sign (and thus, direction) and the detector location:

$$v = (\mu_E + \mu_{EOF})E \quad \text{Eq. 2.4-9}$$

From the above equation, it is apparent that EOF also allows neutral molecules (with a net charge of zero) to be driven towards the detector with a mobility of μ_{EOF} (assuming the detector is between the analyte and anode). Likewise, anions with electrophoretic mobility less than the electro-osmotic mobility will also be driven towards the anode and detector, allowing individual cations, all neutral species, and individual anions to be detected in the same electropherogram with adequate EOF. This further denotes the ability to use EOF as a pumping method within the capillary.

It can be shown that the peak resolution of an electrophoretic system is proportional to the applied electrical field times the square root of the time the analyte stays in the system. Cations move with the electroosmotic flow, and hence their residence time in the system and hence the resolution is decreased by EOF. To prevent this, the channel surfaces are usually coated with a polymer that increases the viscosity at the channel wall and thus suppresses EOF. Throughout this thesis 0.01% (w/v) (hydroxypropyl)methyl cellulose (HPMC) was added to the BGE for this purpose.

2.5 | Conductivity Detection

The mobility matching that leads to a minimal electrodispersion as mentioned above, unfortunately is detrimental to conductometric detection when strong ions are used for the background electrolyte. If the analyte zone experiences the same electric field as the background electrolyte, the specific zone conductivities will be the same (i.e. $\kappa_j = \kappa_{BGE}$) via equation 2.3-1 and a conductivity meter will not be able to detect the analyte. This can be illustrated mathematically via¹⁴:

$$\Pi = \frac{\kappa_j}{c_j} = F \left(1 - \frac{\mu_{counter}}{\mu_j} \right) (\mu_{co-ion} - \mu_j) \quad \text{Eq. 2.5-1}$$

where Π is the molar response of a conductivity detector to an analyte zone of conductivity κ_j (C/V·m·s) and concentration c_j (in mM), F is Faraday's constant, and μ is the effective mobility of the subscripted constituents ($\text{m}^2/\text{V}\cdot\text{s}$). To achieve high responses and better detection, the counter-ion mobility should be much smaller than the analyte's, and the co-ion's mobility should be either significantly larger or smaller than the analyte's. However, the larger the relative mobility, r_j , the more significant the electrodispersive effects. Thus, for conductometric detection, a trade-off exists between peak shape and detector response strength when strong ions are used for the background electrolyte. If weak ions are used, this limitation can be avoided in some instances.

One might think to use a BGE with two co-ions – each having a mobility on either side of the analyte of interest – to minimize the electrodispersion. Unfortunately, in reality this can actually lead to even more disruption of the analyte zone, as will be discussed later.

2.6 | BGEs for Inorganic Cations

2.6.1 | CE buffers for inorganic cations

Our analytes of interest are inorganic cations found in human blood; namely, Na^+ , Mg^{2+} , Ca^{2+} , and K^+ . Furthermore, since lithium is used for therapeutic uses, it is also included in our analyte list. For reference during this section, Table 2.6-1 contains ionic mobilities and pK_a for these cations (taken from Simul 5's database).

Table 2.6-1. Ionic mobilities and pK_a of selected inorganic cation analytes (Simul 5).

Ion	μ ($10^{-5} \text{ cm}^2/\text{V}\cdot\text{s}$)	pK_a
Li^+	40.1	13.8
Na^+	51.9	13.0
Mg^{2+}	55.0	12.2
Ca^{2+}	61.7	13.6
K^+	76.2	13.0

Conductivity detection

Conductivity detection is advantageous in that it can measure both absorbing and non-absorbing analytes simultaneously, and can be readily scaled onto microchip formats. However, as explained earlier and seen in equations 2.4-6 & 2.5-1, analyte zones with the same mobility as the BGE co-ion will be undetectable. This required the development of new background electrolytes to maximize detectability while minimizing electrodispersion.

The MES-His system is the most widely used BGE for conductivity detection of alkali and alkaline earth metals and ammonium ions, although a few other systems for inorganic cation analysis do exist.

Table 2.6-2. System properties of 2-(N-morpholino)ethanesulfonic acid (MES) - histidine (His) background electrolytes.

	Ion	μ ($\text{cm}^2/\text{V}\cdot\text{s}$) ^[7]	pK_a ^[7]	Other Properties	
Co-ion(s)	His^+	26.8×10^{-5}	6.04	pH	~6.0
Counter-ion(s)	His^-	28.3×10^{-5}	9.33	$I^{[17]}$	10.2
	MES^-	28.0×10^{-5}	6.10	(20mM, pH 6.1)	

2-(N-morpholino)ethanesulfonic acid, or MES, is the first of twelve compounds identified by Good, N. et al.¹⁸ as being optimal for biology-related research due to, among other required properties, its inexpensiveness, near-neutral pK_a , stability, low ionic strength, and high tolerance to environmental factors such as temperature and concentration. While its properties make it an excellent buffer, it is not suitable as a background electrolyte due to its low ionic strength.

On the other hand, His is an ampholyte, zwitterion, or "inner salt": a molecule that possesses both a positive and negative charge simultaneously in different places on the structure. This makes it an excellent electrolyte with high ionic strength, but low conductivity due to its net neutral charge. Unfortunately, its net charge is very sensitive to small pH changes around its pK_a value of 6.04.

As we described at the beginning of this chapter, BGEs need to possess both good electrolytic and buffering properties; neither of which MES or His does alone. However, a prepared solution of both species fulfills the BGE requirements, has low conductance, and possesses a co-ion with electrophoretic mobility much smaller than inorganic cation analytes. This makes the MES-His system useful for the analysis of inorganic cations by capillary electrophoresis, which explains why it is the most widely used BGE for conductivity detection in the literature.

Typical concentrations used are 10mM MES and 10mM His for 50 μ m diameter capillaries, and 5mM for 75 μ m¹⁹.

Complexing agents for inorganic cations

Charged compounds that form complexes with the analyte cations can be used to modify their electrophoretic mobility and increase resolution, because the ions will move with an effective mobility that is the weighted mean of the mobilities of their complexed and uncomplexed forms (under the condition that the rate constants for complexation are sufficiently high).

18-crown-6

Francois, C. et al.²⁰ studied the effects of 18-crown-6 on the resolution of inorganic cations in-depth, using a 10mM imidazole BGE titrated to pH 4.5 with acetic acid. They investigated how the crown ether improves the separation of co-migrating K-NH₄ and Sr-Ba ions, and affects the separation efficiency when 0.01mM-0.3M of 18-crown-6 is added. An overview of the effective electrophoretic mobilities of our cations of interest can be seen in Table 2.6-3 (21 different concentrations tested in total).

Table 2.6-3. Overview of 18-Crown-6 influence on some inorganic cations [reproduced].

[18-Crown-6] (mM)	Effective Electrophoretic Mobility ($\mu \times 10^{-4} \text{ cm}^2/\text{V}\cdot\text{s}$)					
	K^+	NH_4	Ca^{2+}	Na^+	Mg^{2+}	Li^+
0.01	6.77	6.77	5.06	4.73	4.57	3.71
1	6.48	6.81	5.09	4.76	4.60	3.74
5	5.39	6.63	5.05	4.71	4.60	3.73
10	4.60	6.43	5.01	4.65	4.60	3.73
50	3.012	5.32	4.69	4.23	4.49	3.65
100	2.45	4.26	4.30	3.72	4.34	3.53

Sulfate

The sulfate ion also affects the mobilities of some inorganic cations. To determine this influence, Havel, J. et al.²¹ substituted perchlorate with sulfate in 1mM steps from 0-4mM at a pH of 3.1. No crown ether was added in this experiment. They determined the addition of sulfate allowed Na and Mg ions to be more effectively separated; however, both Sr and Ca ions were affected similarly and their resolution was not improved with the addition of sulfate. It should be noted that perchlorate has a mobility of $69.8 \times 10^{-9} \text{ m}^2/\text{V}\cdot\text{s}$ and charge of -1; thus, the ionic strength was also increased with the addition of sulfate.

With the addition of 18-crown-6, K/ NH_4 and Sr/Ca ions became resolvable, as shown in Table 2.6-4.

Table 2.6-4. Relative migration times of cations (Na=1) in 4mM $\text{CuSO}_4/\text{HCOOH}/18\text{-Crown-6}$ at pH 3.1 [reproduced].

Cation	Rel_Time	Cation	Rel_Time
Cs^+	0.73	Ni^{2+}	1.19
Rb^+	0.75	Li^+	1.20
NH_4^+	0.76	Ba^{2+}	1.24
K^+	0.85	Be^{2+}	1.27
Na^+	1.00	Pb^{2+}	1.34
Ca^{2+}	1.09	Al^{3+}	1.45
Mg^{2+}	1.15	Y^{3+}	1.59
CO^{2+}	1.17	La^{3+}	1.64
Sr^{2+}	1.18	In^{3+}	1.77
Zn^{2+}	1.18	Fe^{2+}	1.92
Mn^{2+}	1.18		

2.7 | Internal Standards

Microchip capillary electrophoresis (MCE) methods need to be robust as the sample must be analysed correctly not only from run-to-run on a singular chip, but also across multiple chips. Figure 2.7-1 gives an overview of the different sources of variation in MCE. Variation between chips poses a great challenge for quantitative analyses and not much information on it is available in the literature²². Using internal standards (ISTDs) is one possibility to improve quantification of a pre-existing system, including chip-to-chip variation, with no required changes to the physical set-up itself. For these reasons, it is of great interest to determine the significance of chip-to-chip imprecision in MCE and how ISTDs account for it.

Figure 2.7-1. An overview of the different sources of variation in microchip capillary electrophoresis.

Conventional capillary electrophoresis itself has a poor reputation for analytical precision; particularly when it is used with electrokinetic injection (EKI) methods due to the mobility and sample matrix biases, which we will discuss. As a result of significant improvements in pneumatic control, hydrodynamic injection is currently the preferred sample introduction method due to its superior analytical precision^{23–27}. Although there has been some research on developing hydrodynamic injection methods in microchips^{28–30}, EKI still has a distinct advantage with its simplicity. Since high voltage power supplies are already required to perform electrophoretic separations, additional hardware for sample

injections is not necessary – and this is attractive for portable applications that require miniaturised and lightweight electronic systems. EKI also provides preferential loading of analytes with higher mobilities. This selectivity is an advantage when analysing small ions with fast migration times in complex samples such as blood, since additional sample preparation steps are not necessary to prevent larger molecules from impeding the measurement².

The EKI mobility bias results in different amounts of analyte being injected into the system based on the ion's mobility. Unlike CE, this can theoretically be avoided in microchip EKI, provided that the sample is injected long enough and the analyte with the highest mobility is not permitted to diffuse or "leak" into the separation channel; thus, sample focusing (or pinched injection) modes are used. By utilising all four electric potentials and defining the sample plug shape in the intersection, the amount of injected analyte ideally becomes fixed and independent of time³¹. Unfortunately, research has shown that while pinched injection modes do minimise the mobility bias, they do not completely eliminate it; furthermore, a more tightly defined sample plug (i.e. stronger pinching electric fields) shows more bias than a wider sample plug³².

The second bias that EKI methods are prone to in conventional CE systems is the sample matrix bias: different amounts of analyte are injected into the system based on variations in the sample. Changes in properties such as pH, viscosity and ionic strength from sample-to-sample lead to different effective mobilities, and thus, different amounts of loaded analytes. In microchips, the sample reservoir is typically on the order of microliters and applying high voltages on these small volumes can also cause pH changes and sample degradation over the course of a single injection²². Differing ionic strengths of the sample solution versus the BGE also have been shown to affect the sample plug volume formed with pinched injection³³.

Thus, the mobility and sample matrix biases are also points of concern in MCE systems using EKI methods, but in different ways from conventional CE instruments. MCE systems aimed at portability with disposable devices then have the additional concern of reproducibly fabricated microchips. This includes: the channel dimensions, which also contribute to variations in the intersection (or plug shaping) region of the chip; surface properties such as charge, chemistry and roughness; and, variations in the detector alignment. All of these factors can lead to different loaded analyte amounts in the separation channel and/or different detector responses per chip for the same amount of analyte.

2.8 | Conclusion

This chapter has provided an overview of the most important characteristics of capillary electrophoresis, to serve as introduction to and the background for the subsequent chapters.

2.9 | References

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CHAPTER 3

improving chip-to-chip precision in disposable MCE devices with
internal standards^{*}

3.1 | Introduction

In analytical chemistry, an ISTD can be used to improve the repeatability of assay factors which are otherwise uncontrollable, such as sample preparation errors and sample matrix effects. In CE, normalised peak areas (also known as the peak area ratio, PAR) are typically how an ISTD is used to improve quantification. This ratio can be seen in Eq.3.1-1, where PA_A is the peak area of the analyte peak and PA_{IS} is the peak area of the ISTD peak.

$$PAR = \frac{PA_A}{PA_{IS}} \quad \text{Eq. 3.1-1}$$

A propagation of error analysis on this equation yields:

$$\left(\frac{\sigma_{PAR}}{PAR}\right)^2 = \left(\frac{\sigma_A}{PA_A}\right)^2 + \left(\frac{\sigma_{IS}}{PA_{IS}}\right)^2 - \frac{2\sigma_A\sigma_{IS}}{PA_A PA_{IS}} \rho_{A:IS} \quad \text{Eq. 3.1-2}$$

^{*} adapted from Bidulock, A.C.E., van den Berg, A., Eijkel, J.C.T., Electrophoresis 2015, 36, 875-883.

where σ is the standard deviation of the PAR, analyte peak area and ISTD peak area, and ρ_{A-IS} is the correlation coefficient of the two peak areas. The correlation coefficient is the measure of dependence between PA_A and PA_{IS} , where ± 1 is complete correlation and 0 is no correlation. We then set the condition that the relative standard deviation (RSD) of the PAR must be less than the RSD of the analyte (i.e. the ISTD must improve the precision of the measurement):

$$\frac{\sigma_{PAR}}{PAR} < \frac{\sigma_A}{PA_A}$$

and substitute in our propagation of error to get a condition for our ISTD repeatability (Eq.3.1-3):

$$\left(\frac{\sigma_A}{PA_A}\right)^2 + \left(\frac{\sigma_{IS}}{PA_{IS}}\right)^2 - \frac{2\sigma_A\sigma_{IS}}{PA_A PA_{IS}}\rho_{A-IS} < \left(\frac{\sigma_A}{PA_A}\right)^2$$

$$\frac{\sigma_{IS}}{PA_{IS}} < 2 \frac{\sigma_A}{PA_A} \rho_{A-IS}$$

$$RSD_{IS} < 2 \cdot RSD_A \cdot \rho_{A-IS} \quad \text{Eq. 3.1-3}$$

We see that if the peak area of the ISTD is fully correlated with the analyte ($\rho = 1$), the RSD of the ISTD can be up to twice that of the analyte peak area and still improve the variability (to some degree). However, if the peak area of the ISTD is not correlated with the analyte at all ($\rho = 0$), the ISTD will not help as expected. Thus we can see mathematically what we already know intuitively: how well an ISTD accounts for error in the analyte quantification depends both on its own variability and how well it is matched to the analyte.

The use of a single internal standard for improving precision in conventional capillary electrophoresis with hydrodynamic injection has been well investigated and shown to improve intra-column RSDs to less than 1%¹. Two ISTDs have also been verified to correct for run-to-run variation with EKI methods in conventional CE^{2,3}, as well the application of an ISTD to correct for overlapping peaks⁴. While ISTDs have been employed in some MCE applications^{5,6}, minimal investigation has been done on the effect of ISTDs on chip-to-chip variability. Studies which explore chip-to-chip differences typically focus on the repeatability of the fabrication method, such as a related paper which investigated

injection molded PMMA chips using a singular analyte concentration⁷. To the best of our knowledge, a thorough system investigation of how ISTDs affect chip-to-chip reproducibility across a range of analyte concentrations for MCE systems was missing from the literature. The goal of this chapter was thus to investigate if an ISTD is able to account for poor quantification as a result of the chip-to-chip variability inherent in portable MCE systems with single-use microchips.

Table 3.1-1 summarises various factors which can lead to chip-to-chip imprecision in MCE and whether a well-chosen ISTD might correct for each. It can be seen that the amount of sample loaded into the system (channel/injector dimensions, which also influence mobility bias in the sample plug⁸) and the sample detection (detector alignment, which influences its signal response) are possible sources of error between microchips. Since the very definition of a precise assay is the ability to detect the same amount of analyte from the same sample with the same signal response, this poses a great challenge for quantitative analyses.

Table 3.1-1. Some possible sources of chip-to-chip imprecision in a MCE system focused on one-use microchips, if an ISTD is expected to correct for it, and other variations it may contribute to.

Possible chip-to-chip Imprecision source	ISTD improves?	Also contributes to Imprecision in
detection system noise	no	intra-chip (run-to-run)
detector alignment/response	yes	--
channel/injector volume	yes	--
mobility bias (sample plug)	maybe	intra-chip, sample-to-sample
surface chemistry	maybe	intra-chip

To determine the significance of chip-to-chip imprecision in MCE and how ISTDs account for it, we decided to explore the effects of two ISTDs (CsCl and LiCl) on the repeatability and reproducibility of Na quantification across six glass microchips with double-T injectors and contact conductivity detection. Five concentrations of NaCl mixed with running buffer were analyzed six times each with five electrophoretic runs on six microchips, resulting in a dataset of 900 electropherograms to thoroughly determine how both ISTDs perform. We note that this paper is not focused on optimisation of a pre-existing, unique system; rather, a very basic MCE design with common BGE and electrophoretic protocols was used to fully illustrate an ISTD's potential for improving quantification across multiple microchips.

3.2 | Materials and Methods

3.2.1 | microfabricated CE chip

Borofloat glass CE chips as shown in Figure 3.2-1 were fabricated in-house using the same design and principles as described in previous work⁹. In brief, the fluidic channels were etched to a depth of $6\mu\text{m}$ and a width of $52\mu\text{m}$ in the 1.1mm thick top glass plate. Conductivity electrodes are recessed in the 0.5mm bottom glass plate and consist of a $\sim 140\text{nm}$ platinum thin film with a 25nm tantalum adhesion layer. The chip is designed so that the detection electrodes are in contact with the fluid and they are located 20mm from the $200\mu\text{m}$ long double-T intersection. To limit oxidization of the tantalum¹⁰ during the bonding process, the glass chips were annealed in an oven filled with nitrogen gas. Each chip has dimensions of $15 \times 30 \times 1.6\text{mm}$.

Figure 3.2-1. Image of a fabricated glass-glass capillary electrophoresis chip with dimensions of $15 \times 30 \times 1.6\text{mm}$. Reservoir 1 (denoted R₁) is the sample inlet, R₂ is the BGE inlet, R₃ is the sample waste and R₄ is the BGE outlet. The conductivity electrode touchpads for connection with the spring-loaded pins are unlabelled.

After fabrication and dicing, the microchannels were cleaned by flowing 0.1N NaOH through each chip for 10min and then flushing with Milli-Q water for 15min. The channels were then filled with BGE and the glass surface was left to acclimatise¹¹ to the solution for 90 minutes. Milli-Q water was then flushed through the channels again and chips were finally stored in a 50mL conical tube filled with Milli-Q water.

Six chips were chosen from three batches (wafers) fabricated for this chapter: two chips from each batch, each chip from different positions on the 5 by 2 array, and all shown to have similar baseline trends when a test electropherogram was run. Chips on the outer

edge of the round wafer will have thinner conductivity electrodes than those positioned in the middle due to the sputtering process. Separate batches may also have varying channel depths due to slightly different exposure times to and/or concentrations of the glass etchant. Thus, batch and position numbers (i.e. batch#–position#) are used to differentiate intra-chip data when relevant.

3.2.2 | MCE system

A three-piece Delrin® holder consisting of a bottom support plate, a cover with reservoirs, and a top plate was designed for this thesis. The top plate interfaces the fluidic reservoirs to the high voltage supply (CU 411, IBIS Technologies, Hengelo, The Netherlands) via four platinum wires and the conductivity electrodes to the detector electronics via two spring-loaded pins.

Pinched injection is first performed by applying 1000V to the sample inlet (R1) and BGE outlet (R4), 800V to the BGE inlet (R2) and 0V to the sample waste reservoir (R4). The separation step then applies 1000V to the BGE inlet, 600V to the sample inlet and waste, and 0V to the BGE outlet. The first injection time for a chip load is 60s; all subsequent injection and separation times are both 45s.

An Arduino Uno microcontroller board and high vacuum reed switches isolated the detection electronics from the microchip during the pinched sample injection, preventing DC current leakage into the thin film electrodes. During separation, the detector electronics apply a 30kHz 0.75V_{rms} AC voltage signal. The voltage drop across the MCE system is measured using a simple voltage divider and lock-in amplifier circuitry, which effectively measures the change in conductivity between the two thin film electrodes. While both magnitude and phase responses are collected by the system, only the magnitude is used for signal processing. The detection electronics and accompanying software used to control and record all signals were developed in-house.

Electrophoretic data was collected at 238.42Hz and subsequently processed using a custom-made MATLAB script. A Butterworth low-pass filter with a cut-off frequency of 5Hz was applied to the collected data and then a moving median subtraction of 175 points (corresponding to a width of 0.734s) was used to remove the signal's baseline. Peak areas used a triangle approximation by multiplying the peak height by the full width at half maximum (FWHM) as this was found to be more reproducible than integrating the area. The migration time was then taken as the half maximum time on the rising edge of the peak.

3.2.3 | reagents

BGE stock solutions of 250mM MES hydrate and 250mM L-histidine (His), 0.05% (w/v) (hydroxypropyl)methyl cellulose (HPMC), and 200mM KCl were prepared prior to the experiment set. Aliquots from stock were diluted and mixed together daily to form a running buffer of 100mM MES-His, 0.01% (w/v) HPMC and 0.25mM KCl. After preparation, the BGE had a measured pH of 6.135 at 22.2°C. HPMC was added to the BGE as an EOF modifier, in the same concentration as shown to successively reduce the EOF in previous work¹². KCl was added to the BGE to reduce interactions of Cs and Na with the glass and improve peak shape, as reported on previously¹³ (also see Figure 3.2-2).

Figure 3.2-2. Electropherograms investigating the peak shapes of Cs and Na with increasing KCl concentration added to the background electrolyte. Sample consisted of 5mM CsCl or 5mM NaCl in BGE (100mM MES-His, 0.01% HPMC) and 0mM, 0.10mM or 0.25mM of KCl in BGE. The peak shape is noticeably improved even with the addition of 0.10mM of KCl, but the Cs peak still has a distinct leading and trailing edge. This is further improved when the concentration is increased to 0.25mM KCl.

Sample solutions were also prepared from stock daily and contained 100mM MES-His, 0.01% HPMC, 7.5mM of each ISTD (CsCl and LiCl), and one of five different concentrations of NaCl (2, 4, 6, 8 and 10mM). The same concentration of MES-His was used in both the sample and BGE solutions to limit sample stacking effects over the analyte concentration range.

The LiCl stock solution was graciously donated by Medimate (Enschede, The Netherlands); all other chloride salts and BGE chemicals were 99% grade or higher and were purchased from Sigma-Aldrich (Steinheim, Germany). Milli-Q water was used to dissolve all reagents and dilute all stock solutions.

3.2.4 | experimental procedure

An electrophoretic "run" is defined as one injection-separation pair. A chip "load" is defined as all electrophoretic runs applied to a chip containing the same aliquots of BGE and sample solutions. Each chip load consisted of 5 electrophoretic runs. For each sample solution of different NaCl concentration, 6 chip loads were performed per chip. Thus, 6 microfluidic chips x 5 analyte concentrations x 6 chip loads x 5 runs resulted in 900 electropherograms for this data set.

Two chip holders were built so experiments could be performed in tandem. To clean and refill the microchip after an experiment, all reservoirs were first rinsed three times with Milli-Q water. The buffer outlet was then rinsed three times with BGE and subsequently filled with BGE. Vacuum was applied to the other three reservoirs for the length of the experiment on the second chip; approximately 10 minutes. This effectively flushed new buffer through the channels of the chip, displacing the waste into the three empty reservoirs. After removing the vacuum, these reservoirs were emptied and rinsed three times with BGE. 55 μ L of BGE was then pipetted into the buffer inlet, outlet and sample waste reservoirs. Lastly, 55 μ L of sample solution was pipetted into the sample inlet reservoir immediately prior to running the next experiment.

3.3 | Results and Discussion

3.3.1 | thin-film electrode characterisation

After the chips were fabricated, cleaned and introduced to the BGE, impedance sweeps were performed on the detector electrodes using a Hewlett Packard 4194A Impedance/Gain-Phase Analyzer to determine the optimal conductivity measurement frequency. After cleaning with NaOH, the six chips were filled with BGE and 51 impedance spectra per chip were taken from 100Hz to 40MHz. The first measurement of each chip

was set aside and the remaining 50 were averaged for the final chip characterisation. At 30kHz, the six chips had magnitude and phase responses of $0.372\text{M}\Omega \pm 1.95\%$ and $-14.9^\circ \pm 7.78\%$, respectively. Individual chip characterisations can be seen in Figure 3.3-1.

Figure 3.3-1. Magnitude and phase values for each microfluidic chip's conductivity detection electrodes (including the chip holder's connections) at 30kHz. Impedance sweeps were performed using the HP 4194A Impedance/Gain-Phase Analyzer and 50 measurements were averaged to characterise each chip.

3.3.2 | internal standards

Lithium and cesium were chosen as ISTDs because they do not occur naturally in most samples and are often used as ISTDs in flame spectroscopy (cesium can be used when the patient takes lithium therapeutically to determine sodium, potassium and lithium¹⁴). Furthermore, the electrophoretic mobilities of cesium and lithium bracket many other cations of interest, including sodium, magnesium and calcium.

The chosen ISTD should have good correlation with the analyte(s), as indicated in Eq.3. To increase the correlation, the standard concentration should result in a peak area and shape that is similar to the analyte. It should further have a good RSD. Three concentrations of CsCl and LiCl (5mM, 7.5mM and 10mM) were loaded on one microchip five times per concentration ($n=25$). In increasing concentration, the RSDs of LiCl were 3.49%, 2.37% and 2.93%, and the RSDs of CsCl were 2.55%, 2.19% and 2.66%. All peaks

had similar peak shape, so 7.5mM was chosen for both ISTDs as it had the lowest variation.

3.3.3 | reproducibility of migration time

While intra-chip migration time RSDs for Na were approximately 1.5% on average, chip-to-chip variation increased this value to 3.60%. Chip-to-chip RSDs for Cs and Li were 2.33% and 4.07%, respectively. Thus, chip-to-chip variation increased with retention time, as illustrated in Figure 3.3-2a. This could indicate: a) differences in separation length due to variation in the conductivity electrode alignment (separation path lengths); and/or, b) incomplete suppression of an EOF which shows chip-to-chip variability due to differences in chip surface properties. This trend of larger RSDs for peaks with higher migration times is not reflected in the intra-chip data; here, the analyte peak was typically the most variable. This suggests that there was minimal to no intra-chip EOF variation. The precision of intra-chip and chip-to-chip migration times for all measurements are given in Table 3.3-1.

Table 3.3-1. Summary of migration time RSDs for all experiments.

	Na	'Cs'	'Li'	Na/'Cs'	Na/'Li'	Na (new)
Chip 1-3	2.99%	2.09%	2.86%	0.98%	0.45%	0.58%
Chip 1-9	0.89%	0.82%	0.94%	0.52%	0.64%	0.64%
Chip 3-2	1.43%	0.81%	1.08%	0.70%	0.47%	0.53%
Chip 3-3	1.48%	0.81%	1.13%	0.77%	0.50%	0.57%
Chip 4-1	1.50%	1.14%	1.41%	0.72%	0.50%	0.54%
Chip 4-7	3.21%	1.98%	3.29%	1.33%	0.34%	0.46%
All	3.60%	2.33%	4.07%	1.76%	0.82%	0.57%
Average	8.76 s	6.41 s	10.6 s	--	--	8.59 s

When the Cs peak was used as an ISTD, the chip-to-chip migration time ratio for Na improved from 3.60% to 1.76%; with the Li ISTD peak, it improved to 0.82%. Thus, with respect to the migration time, the Li peak shows a higher correlation to the Na peak than Cs does. This makes it the better single ISTD for migration times, even though the chip-to-chip migration time of Li is less precise than Na and Cs.

We also investigated using both the Cs and Li peaks to correct the Na migration time. Keeping the relative distance between the three peaks the same, the Cs and Li peaks were

Figure 3.3-2. One electropherogram taken from each chip's analysis of 6mM NaCl, 7.5mM CsCl and LiCl. In (a) time scales are unadjusted, while in (b) peaks are shifted based on Eq.4. Migration time RSDs for each peak are given on the figure. Electropherograms are drawn post-processing. Negative baselines to the right of peaks are inherent to the raw data. We attribute these "dips" to electrochemical effects at the thin-film conductivity electrodes due to their chip-specific nature and observed independence from the peak area.

adjusted to their average values, and the new Na migration time was then calculated. This can be represented by the following equation:

$$\frac{t_{Na} - t_{Cs}}{t_{Li} - t_{Na}} = \frac{x - t_{Cs,avg}}{t_{Li,avg} - x}$$

$$x = \frac{t_{Cs,avg}(t_{Li} - t_{Na}) + t_{Li,avg}(t_{Na} - t_{Cs})}{t_{Li} - t_{Cs}} \quad \text{Eq. 3.3-1}$$

where x is the new Na migration time, and $t_{Cs,avg}$ and $t_{Li,avg}$ are the average migration times of Cs and Li, respectively. Figure 3.3-2 shows six electropherograms (one from each chip), before and after the dual ISTD migration time adjustment, with the peaks noticeably more aligned. This method further improves the chip-to-chip variation to 0.57%, making it more reproducible than a single ISTD.

3.3.4 | linearity of peak area

Calibration curves were built by performing a standard linear regression on the five concentrations of NaCl analysed, first for each chip and then for all chips. Considering only Na peak areas, all chips but one gave good intra-chip correlation coefficients (R^2) of better than 0.99, indicating excellent intra-chip linearity. Intra-chip coefficients are provided in Table 3.3-2. The chip-to-chip correlation, however, was only 0.943. This decrease in linearity is due to varying slopes and intercepts for each chip, whereby chips with higher peak areas also show steeper slopes (Figure 3.3-3a). Chip-to-chip linearity therefore is less. When Cs is used as an ISTD, both intercepts and slopes of the intra-chip calibration curves align (Figure 3.3-3b), and the chip-to-chip linearity improves to $R^2 = 0.991$. In the Na/Li plot however, two distinct groups of chips with like intercepts and slopes are observed (Figure 3.3-3c), which only improves the linearity to $R^2 = 0.971$. Thus, Cs is the better ISTD.

Table 3.3-2. Summary of linear trend fitting for the Na calibration curves.

	R^2_{Na}	$R^2_{Na/Cs}$	$R^2_{Na/Li}$
Chip 1-3	0.966	0.995	0.992
Chip 1-9	0.994	0.993	0.992
Chip 3-2	0.999	0.999	0.999
Chip 3-3	0.998	0.999	0.998
Chip 4-1	0.993	0.996	0.994
Chip 4-7	0.993	0.997	0.995
All	0.943	0.991	0.971

Figure 3.3-3. Linearity data for intra-chip calibration curves: (a) Na peak areas only, (b) Na/Cs PAR, and (c) Na/Li PAR. Error bars indicate one standard deviation of six measurements from one chip.

3.3.5 | reproducibility of peak area

Figure 3.3-4a compares the Na peak area RSDs at each concentration, first for each microfluidic chip and finally across all chips. The overall trend of the data suggests that the smaller NaCl sample concentrations (i.e. 2mM and 4mM) have larger variability. Outliers with larger RSD values (e.g. 8mM on Chip 1-3) are neither confined to one chip, batch nor day, and vary from concentration to concentration. Chip 3-3 was consistently the most reproducible with RSDs of <3%, and along with the other in its batch (Chip 3-2), also had the best linearity and migration time repeatability. However, Chip 3-2 has one of the larger Na peak area RSDs for every analyte concentration analysed, so the good performance of Chip 3-3 does not seem to be batch related. Neither does it seem to be position related as Chip 1-3 is one of the most variable chips. Figure 3.3-4a also illustrates that the chip-to-chip variation of the Na peak is noticeably larger than the intra-chip variation, as expected. RSD values are approximately 10% for higher NaCl concentrations (6mM-10mM), and about 16% for lower NaCl concentrations (2mM, 4mM).

The variation in magnitude response of the thin-film detector electrodes was approximately 2%. Since the detector circuitry uses a voltage divider configuration, this variation would be halved with a perfectly matched resistor. In Figure 3.3-4b, the chip-to-chip variability of the Na peak (Na) and Na peak scaled by the normalised magnitude response ($\text{Na} \cdot Z$) are shown. As expected, the precision improves by approximately 1%.

Variation in peak retention time due to differences in velocity results in varying peak widths, corresponding to the speed at which they pass the detector. Similarly, differences in detector distance from the separation channel results in different peak widths due to diffusion; the longer the peak is retained, the more it diffuses. Normalising the Na peak by its migration time (Na/t) improves the chip-to-chip variation by about 1.5% at higher NaCl concentrations (6mM-10mM) and about 2.5% at lower (Figure 3.3-4b).

The normalised magnitude response and migration time can further be combined to modify the Na peak area ($\text{Na} \cdot Z/t$ in Figure 3.3-4b). This results in RSD values which are ~7% for 6mM-10mM NaCl and ~14% for 2mM-4mM NaCl – and which are still larger than the intra-chip Na peak area variation.

Figure 3.3-4. Relative standard deviations for each concentration of Na. In (a), the intra-chip RSDs and chip-to-chip RSDs are shown for Na peak areas at each measured concentration; the chip-to-chip are noticeably larger. In (b), chip-to-chip RSDs are shown for: the Na peak areas (Na); peak area scaled by the normalised detector response (Na*Z); peak area normalised with the migration time (Na/t); the previous two combined (Na*Z/t); and, the two internal standard PARs (Na/Cs and Na/Li). The chip-to-chip variations for all Na concentrations are larger than the intra-chip data. While all methods improve the chip-to-chip reproducibility, the Na/Cs PAR is most effective.

Table 3.3-3. Summary of intra-chip and inter-chip relative standard deviations (RSD) for all peak areas and peak area ratios (PAR).

		<i>Chips:</i>	1-3	1-9	3-2	3-3	4-1	4-7	All
2mM	Peak Area RSD [%]	Na	4.84	2.76	5.78	2.93	6.81	4.87	16.0
		Cs	3.59	1.66	0.96	1.42	2.07	2.28	10.8
		Li	3.61	1.37	1.93	1.90	2.38	1.82	6.25
	PAR RSD [%]	Na/Cs	3.60	3.17	2.06	2.40	5.09	3.89	8.81
		Na/Li	3.59	3.31	2.31	2.50	5.26	4.10	13.7
4mM	Peak Area RSD [%]	Na	5.07	5.09	4.63	2.16	2.96	2.81	15.6
		Cs	2.73	4.45	1.49	1.54	1.67	1.44	11.2
		Li	1.72	2.11	1.85	1.50	1.20	1.15	6.10
	PAR RSD [%]	Na/Cs	3.09	2.11	1.71	1.60	2.09	2.36	7.06
		Na/Li	5.98	5.21	1.70	1.68	2.12	2.96	14.2
6mM	Peak Area RSD [%]	Na	1.88	2.39	3.35	2.20	5.53	5.38	9.10
		Cs	2.03	2.44	1.16	1.63	2.09	2.14	8.69
		Li	1.95	1.78	1.94	1.69	1.16	1.86	6.08
	PAR RSD [%]	Na/Cs	1.22	1.55	1.18	1.66	3.90	3.95	4.02
		Na/Li	1.64	2.00	1.63	2.23	5.59	5.25	6.91
8mM	Peak Area RSD [%]	Na	7.25	1.18	2.7	1.27	2.98	2.44	10.2
		Cs	5.95	1.61	1.21	1.00	2.70	2.95	10.0
		Li	7.09	1.45	1.30	0.99	2.07	1.66	6.45
	PAR RSD [%]	Na/Cs	2.13	1.41	1.02	1.42	1.44	1.16	2.51
		Na/Li	1.37	1.70	1.44	1.45	1.60	1.68	6.26
10mM	Peak Area RSD [%]	Na	3.56	2.50	2.77	1.49	1.68	2.73	10.3
		Cs	5.53	5.78	1.28	1.15	1.76	3.20	13.8
		Li	1.23	1.75	1.74	0.84	2.19	2.77	4.98
	PAR RSD [%]	Na/Cs	2.81	3.77	1.11	1.15	1.10	1.28	4.33
		Na/Li	3.60	3.70	1.36	1.97	1.60	1.44	7.25

When the PARs of the ISTDs are used (Na/Cs and Na/Li in Figure 3.3-4b and Table 3.3-3), the chip-to-chip reproducibility also improves. However, unlike the migration time ratios, Li does not improve the chip-to-chip variation as well as Cs. In fact, the Na/Li PAR is not much better than the combined magnitude response and migration time normalisation ($\text{Na} \cdot \text{Z}/t$). The Na/Cs PAR's superiority can also be seen numerically in Table 3.3-4, which gives the peak area and PAR RSDs for all peaks. Using these values and the uncertainty propagation from Eq. 3.1-2, it is possible to calculate the experimental correlation coefficient, ρ , for each Na concentration:

$$\rho_{A \cdot IS} = \frac{PA_A PA_{IS}}{2\sigma_A \sigma_{IS}} \left[\left(\frac{\sigma_A}{PA_A} \right)^2 + \left(\frac{\sigma_{IS}}{PA_{IS}} \right)^2 - \left(\frac{\sigma_{PAR}}{PAR} \right)^2 \right]$$

$$\rho_{A \cdot IS} = \frac{RSD_A^2 + RSD_{IS}^2 - RSD_{PAR}^2}{2RSD_A RSD_{IS}} \quad \text{Eq. 3.3-2}$$

Table 3.3-4. Summary of chip-to-chip RSDs for all peak areas and PARs, with the correlation coefficient for each (calculated using Eq. 5). Values for the intra-chip data were also calculated and can be found in Table 4.

[NaCl]	Peak Area RSD [%]			PAR RSD [%]		Correlation ρ	
	Cs (ISTD)	Li (ISTD)	Na (A)	Na/Cs	Na/Li	Na·Cs	Na·Li
2mM	10.8	6.25	16.0	8.81	13.7	0.854	0.542
4mM	11.2	6.10	15.6	7.06	14.2	0.913	0.411
6mM	8.69	6.08	9.10	4.02	6.91	0.899	0.652
8mM	10.0	6.45	10.2	2.51	6.26	0.969	0.810
10mM	13.8	4.98	10.3	4.33	7.25	0.976	0.766

The calculated correlation coefficients, as given in Table 3.3-4, indicate that the Cs peak area has a higher correlation with the Na peak area – despite the Li peak area being less variable than the Cs peak area. This is because the Li peaks, while more stable, do not follow the trend of the Na peak areas: where Na rises or falls in value, so does the Cs peak area. This can be seen visually in Figure 3.3-5.

We attribute the higher correlation of the Cs peak area to one or both of the following explanations. As mentioned previously, a minute amount of KCl was added to the BGE to reduce interactions of the ions with active sites on the glass surface, as theorised in previous work¹³. The peak shapes that showed the most improvement by the KCl addition were Cs and Na, while Li's peak shape was relatively unchanged. Thus, Cs is likely better

correlated to Na due to these ions interacting with the glass surface more than Li¹³. Another possible explanation is the mobility bias seen in microchips where pinched injection modes are used⁸, whereby ions with higher mobility are selectively (de-)stacked in the intersection based on the width of the pinched sample flow. This flow width would vary from chip-to-chip due to varying intersection volumes. However, since the chip-to-chip RSDs of the Cs peak areas are lower than Na (aside from one concentration), the latter explanation is discounted: if mobility bias was the major contributor, the higher mobility of Cs should lead to a higher RSD.

Figure 3.3-5. Peak area distribution of the calculated Cs, Na and Li peak areas for all experiments analysing 6mM NaCl, 7.5mM CsCl and LiCl. Each grid line on the x-axis represents a new chip load, while each point on the graph represents one electrophoretic run.

3.3.6 | sources of chip-to-chip imprecision

In Table 3.1-1, we gave some possible sources of imprecision in a MCE system focused on disposable point-of-care devices and if we expected an ISTD to correct for each.

Detection system noise. When numerical integration is used, choosing the start and end points of the peak can be crucial and easily influenced by detection system noise. Here, the FWHM and height were used to approximate the peak areas, which should effectively minimise any influence of detection system noise.

Detector alignment/response. The detector response was expected to contribute about 1% to the RSD. This was confirmed in the previous section and accounted for by scaling the Na peak area with the normalised magnitude response of each individual chip. However, this would require the MCE system to be able measure the open-circuit impedance of each chip prior to the measurement, whereas the ISTD PAR should account for this. The detector alignment is expected to primarily impact the migration time, increasing chip-to-chip imprecision with retention time of the peak. This trend was seen in the migration time data, whereby the RSDs of peaks increased as electrophoretic mobility decreased. To correct for this, both ISTDs were used to adjust the Na migration time based on its relative distance to the standard peaks. This improved the migration time significantly; however, using this adjusted time to also adjust the peak areas had no effect on the peak area reproducibility. Normalising the Na peak area with its migration time improved the Na peak areas, but had no effect on the PAR; thus, any variations in peak width due to detector alignment and/or migration velocity are accounted for by the ISTD PAR.

Channel/injector volume. Channel/injector volume would also be accounted for by the ISTD PAR, as all analyte volumes loaded into the system are expected to increase/decrease proportionally. Previously, it was shown that the Na/Li PAR did not improve the Na peak areas much more than the combined magnitude response and migration time normalisation. Thus, considering that the Na/Li PAR should at least account for variations in the detector alignment/response and channel/injector volume, the latter is not expected to contribute significantly to the chip-to-chip variation.

Mobility bias. As explained in the previous section, mobility bias within the sample plug due to the pinched injection mode is not suspected to have been a significant source of error. If it were, the analyte with the fastest mobility (Cs) should have had the highest RSDs, and this only occurred in the 10mM NaCl concentration dataset.

Surface chemistry. Since ions interact with the microchannel glass differently based on properties inherent to each molecule, it is expected that variations in surface chemistry would only improve with a well-chosen ISTD. While both the Cs and Li PAR improve the chip-to-chip precision, it was found that Cs was far superior: further improving RSDs from Li's ~7% to ~4% (6mM-10mM NaCl) and ~14% to ~8% (2mM, 4mM NaCl). We attribute this increased improvement primarily to analyte interactions with the glass surface.

3.4 | Conclusion

None of the point-of-care CE systems found in literature reports on the use of ISTDs to improve chip-to-chip RSDs¹⁵. The goal of this chapter was thus to investigate if an ISTD could improve chip-to-chip precision. It was demonstrated that ISTDs are a viable means to correct for *chip-to-chip* imprecision in disposable, point-of-care MCE devices and improved the RSD by a factor of 2 to 4, depending on the analyte concentration. We showed that, as in other analytical chemistry methods, ISTD substances must be chosen carefully. Both ISTDs explored in this chapter improved the Na peak area reproducibility; however, only one accounted for variation in microchannel surface properties, which was determined to be the main source of chip-to-chip variability. Other chip-to-chip imprecision contributors only made up a few percent of the RSDs, and could be further minimised with industry manufactured microchips over in-house fabrication by an inexperienced operator.

While the RSDs shown in this chapter are not nearly as precise as would be required in a true point-of-care system, it should be noted that non-optimised protocols were used for this study as its purpose was to investigate chip-to-chip reproducibility improvements by addition of an ISTD. In commercial systems, rigorous testing of the electrophoretic method (such as permanent glass coatings, electric fields, injection/separation times, and BGE composition/concentration) would certainly improve the RSDs further.

3.5 | References

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CHAPTER 4

improved quantification for point-of-care capillary electrophoresis by incorporating an internal standard in the background electrolyte^{*}

4.1 | Introduction

Recently, MCE has developed to a promising platform for point-of-care diagnosis of small ions.¹⁻⁴ Addition of ISTD to the sample is a standard technique to improve run-to-run quantitation in conventional CE⁵⁻⁷ and MCE^{8,9}. In the last chapter, we demonstrated that adding an ISTD to the sample not only can be used to improve run-to-run quantitation, but also improve chip-to-chip precision.¹⁰ The latter is crucial when disposable microchips are used as is common in POC analysis. However, in POC analysis, this would require an extra sample preparation step performed by either an unskilled operator or the device itself. Integration of a microfluidic mixing system with the CE microchip would introduce new error sources, increase system complexity, and raise fabrication costs. These are severe limitations for a quantitative, disposable microchip.

As stated in Chapter 2, for a BGE of n constituents, there will be $n-1$ system peaks present. When one of those constituents is present in small amounts relative to the others, its corresponding system peak will move with a migration velocity similar to the added

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ion^{11,12}. While investigating the amount of KCl required in the BGE to improve the analyte (Na^+) peak shapes in Chapter 3.2.3, a negative system peak was observed at approximately the same arrival time a K^+ peak would be expected (Figure 4.1-1). As the concentration of KCl was increased for the same sample and BGE, the peak size increased as well.

Figure 4.1-1. Electropherograms investigating the minimal amount of KCl necessary in the BGE to improve the Na^+ analyte peak shape. Measurements are off-set horizontally by 1 second and vertically by 1 unit to clearly see the improvement in the analyte peak and the appearance of the "K" system peak.

Although this phenomenon is well-known, to the best of our knowledge, the literature does not report on the use of intentionally-added system peaks for the purposes of improving quantitative measurements. The authors speculate that this is due to the complex nature of system peaks and the fact that a traditional ISTD is readily available for use by a skilled technician in a standard laboratory setting. It is only when we try to address the problem of precise measurements in disposable microchips used by unskilled operators that the potential of using a reference system peak instead of a traditional ISTD

becomes attractive: no additional sample manipulation, microchip changes or system expansions are required with this method, which makes it an ideal solution for POC applications.

Here, we present a novel method to improve chip-to-chip precision in disposable MCE devices whereby the reference standard is added to the BGE instead of to the sample. The resulting system peak area or height from the added reference ion is then used to correct for different sources of system error in the quantitative measurement, similar to a traditional ISTD peak. We explore the effects of two reference system peaks (CsCl and LiCl) on chip-to-chip reproducibility of Na quantification, using a dataset of 900 electropherograms collected from measuring five concentrations of NaCl repeatedly on six microchips. The in-house fabricated microchips, CE protocols and reagents were the same as previously presented in Chapter 3, allowing us to draw reliable conclusions about the viability of the new method. We thus compare Sample-added Internal Standards ("Sample IS") to BGE-added Reference Standards ("BGE Ref").

4.2 | Materials and Methods

4.2.1 | MCE system

This experiment set used the same chip holders, electrophoretic methods and equipment as in previous work. For details on the experimental method and setup, please see Chapter 3.2.

The same six microfluidic chips used to investigate Sample-added ISs, were also used for the experiments in this chapter. Between this experiment set and the previous, each microchip was cleaned by flowing 0.1N NaOH and water through the chip for 10min and 15min, respectively. The channels were then filled with BGE solution (sans the added standards) and left for at least 90 minutes so the surface chemistry of the glass could adjust to the solution. For storage, the chips were then flushed with Milli-Q water and kept in a 50mL conical tube also filled with Milli-Q water.

4.2.2 | reagents

BGE solutions were mixed daily from stock solutions and consisted of 100mM MES-His, 0.01% (w/v) HPMC, 0.25mM KCl, and 7.5mM of each added reference standard (CsCl and LiCl). This is the same BGE as used in Chapter 3, only with the addition of the ISTD ions (in the same concentration as previously added to the sample). Sample solutions were also prepared from stock daily and contained 100mM MES-His, 0.01% HPMC, and one of five different concentrations of NaCl (2, 4, 6, 8 and 10mM).

4.2.3 | baseline-fit

In Chapter 3, the collected electrophoretic data was filtered using a Butterworth low-pass filter (with a cut-off frequency of 5Hz) and the signal's baseline was approximated using a moving median subtraction of 175 points (or 0.734s at a data collection rate of 238.42Hz). This estimation was sufficient when all peaks of interest in the electropherogram were positive and the baseline had minimal curvature. In this experiment set, however, the electropherogram contains both negative (BGE-added IS) and positive (analyte) peaks. As illustrated in Figure 4.2-1, the moving median subtraction does a poor job of estimating the baseline across the BGE-added ISTD peaks (particularly for 'Cs'), which affects the repeatability of the measurement.

Figure 4.2-1. Two baseline fits to the same electropherogram. "MedSub" stands for Median Subtraction and was the method used in Chapter 3. "BackCorr" represents the Background Correction script from MATLAB file exchange, with settings of: Huber symmetric function, order 5, threshold 0.01. The polynomial fit more accurately represents the baseline with minimal influence from the peaks.

Thus, this chapter uses a background correction (backcor) method as found on MATLAB's File Exchange and described in literature by V. Mazet, et al.¹³ In short, the script applies a polynomial fit to the background using one of four predefined functions, a user-defined order and a threshold setting which limits the influence of the peaks on the estimation. Here, a symmetric Huber function was used with a polynomial order of 5 and a threshold of 0.01, which fits well to the baseline (Figure 4.2-1). Peak areas are then estimated using the full width at half maximum, and the migration time was taken as the half maximum time on the rising edge of the peak.

4.3 | Results and Discussion

4.3.1 | understanding BGE-added reference peaks

Size and migration times of system peaks are often hard to calculate without the use of advanced mathematical models and/or simulation programs, as mentioned in section 2.3.5 of this thesis. In a simple system, whereby the sample and BGE have similar conductivity and ionic strength, the resultant system peaks are similar in size to the traditional ISTD (Figure 4.3-1a). Since the ratio of CsCl and LiCl concentration (7.5mM) to that of the concentrations of the other BGE constituents (100mM MES-His) is small, the resultant system peaks also have migration times similar to their corresponding sample peaks. An estimate using equation 2.3-10 predicts system peak migration times about 8% smaller than that of sample-added Cs and Li.

BGE-added reference peaks are denoted with quotations because they are not moving analyte zones of Cs or Li, but rather the inverse: they are depleted regions in the BGE where notably less Cs and Li ions are present. When the sample is electrokinetically-injected into the microchip injection channel, the sample (de)stacks as it crosses the stationary boundary into the BGE (see Chapter 2). In the sample plug, prior to separation, there are no Cs or Li ions present – they are completely displaced by the sample zone during EKI. This discontinuity in the BGE (the sample plug) gives rise to the two depleted-ion system zones moving through the separation channel with a migration velocity similar to that of the depleted ion. However, it should be noted that since these are BGE constituents and not sample constituents, these zones are not the only depleted regions for that ion in the system; rather, they are the most significantly depleted for that ion. Figure 4.3-1b, which was produced by the simulation program Simul 5^{14,15}, shows this phenomena. This complex composition of the system peaks stands in contrast with the sample peaks, where each ion has one analyte region, though in this region the concentrations of the BGE ions are also changed.

Figure 4.3-1. (a) Two electropherograms comparing 7.5mM CsCl and LiCl added both in the 6mM NaCl-containing sample (producing positive ion peaks) and in the BGE system (producing negative system peaks). Both sample and BGE contain 100mM MES-His, and thus are similar in conductivity and ionic strength. **(b)** Changes in ion concentrations of moving zones passing the detector in BGE Ref, simulated using Simul 5^{14,15}. As Cs and Li ions are both BGE constituents, their concentrations change for all moving zones in the BGE system, similar to MES and His. However, the two peaks which move with migration velocity similar to Cs and Li can be seen to primarily be changes in Cs and Li concentration.

While the mobilities of system peaks, including our BGE-added standards, are dependent on the BGE composition, the size of the peaks are strongly dependent on both the BGE and the sample matrix¹⁶. Figure 4.3-2a plots five electropherograms collected from the same chip with BGE-added standards, each measuring a different Na concentration. As the concentration of Na increases in the sample – also increasing the sample's conductivity and ionic strength – the 'Cs' system peak height becomes more negative and the 'Li' system peak less. This conflicts with the idea of a true internal standard: a substance which is different from the analyte of interest, but is influenced by the sample preparation and measurement system in the same way.

A true internal standard, however, cannot exist in MCE devices which use EKI methods. In Chapter 2, we stated that the amount of injected analyte ideally is fixed and independent of time, provided that the sample is injected long enough and is restricted from diffusing into the separation channel. In practice however, a mobility bias still exists. While the injected ions are assumed to have the same concentration ratio with respect to each other, other constituents and properties of the sample will influence the accuracy of that ratio. Pinching the sample in the injection phase, while preventing diffusion, can also lead to stacking of ions with faster mobilities in the sample plug. Figure 4.3-2b similarly plots five electropherograms collected from the same chip with Sample-added ISs, and a slight decrease in the internal standard peak height is visible as the NaCl concentration increases.

In spite of these sources of variability, Chapter 3 clearly demonstrated that Sample-added ISs are a means to improve precision in MCE. Thus, it is also of interest to determine if BGE-added reference standards can effectively improve the precision of the measurement, despite their complex relationship with the sample and BGE matrices. This chapter reports the results of this investigation.

Figure 4.3-2. (a) Five electropherograms collected from the same chip with BGE-added standards, each measuring a different Na concentration. **(b)** five electropherograms collected from the same chip with Sample-added ISs, each measuring a different Na⁺ concentration. In both cases a change in the internal standard height is observed as function of the sample Na⁺ concentration.

4.3.2 | simulation of the injection phase: simulating varying chip dimensions

Apart from differences in BGE and sample matrices, it is also important to know how BGE Ref peaks are influenced by varying channel dimensions and if they can account for physical chip-to-chip differences. Since we could not experimentally verify this, we investigated this using the 2-dimensional simulation program Simul 5, by simulating the same injected sample with different plug widths. The double-T intersection has a length of 0.2mm; ignoring pinching effects, six other widths are investigated between 0.15-0.25mm.

As expected, the Na analyte peak area increases with sample plug width: the more sample present, the more Na ions are also present. Likewise, the BGE Ref peaks also increase in peak area with plug width: the more BGE that is displaced, the larger the moving zones of missing Cs/Li ions are. The rate of change for each peak, however, is different (Figure 4.3-3a). With a true internal standard, the rate of change would be equal for both analyte and ISTD peaks, leading to slope of zero at all plug widths when the PAR is taken. Due to the varying slopes, the PARs in the BGE Ref simulations also have slopes (Figure 4.3-3b). Although the resulting PAR is actually a rational function, it can be approximated as a linear line with slope and intercept given in Table 1. The slopes of both the Na/'Cs' and Na/'Li' PARs are about 10% of Na's; in other words, they are a factor of $1/10^{\text{th}}$ less sensitive to changes in channel dimensions than the Na peak area is alone. This means that while the BGE Ref peaks do not behave like an ideal internal standard when considering varying chip dimensions, these PARs can still in good approximation be used to correct the Na peak area.

Table 4.3-1. Slopes and y-intercepts of the linear regressions imaged in Figure 4.3-3.

	Slope [units/mm]	y-intercept [units]
Na	2.09	-0.0083
'Cs'	1.78	0.0075
'Li'	1.34	0.0017
Na/'Cs'	0.232	1.08
Na/'Li'	0.212	1.48

Figure 4.3-3. (a) The peak areas of the Na analyte peak as well as the BGE Ref peaks 'Cs' and 'Li' increase with sample plug width. (b) The PAR of Na and both BGE Refs remains constant within 10% on changing the sample plug width, indicating successful correction for the sample plug width.

4.3.3 | chip experiments: reproducibility of migration time

As in the "Sample IS" experiment set reported in Chapter 3, chip-to-chip migration time RSDs were larger than intra-chip variations: 2.93%, 2.34% and 3.69% for Na, Cs and Li, respectively. These values for "BGE Ref" are very close to those found in the previous chapter, with the Na variability somewhat superior here. Imprecision in chip-to-chip RSDs again increased with retention time, indicating a) variation in separation path lengths due to varying conductivity electrode alignment; and/or, b) variation in chip-to-chip EOF due to differences in chip surface chemistry. Intra-chip and chip-to-chip RSDs for "BGE Ref" are given in Table 4.3-2, and compared to chip-to-chip values for "Sample IS".

Using "BGE Ref", the migration time reproducibility of Na can be improved with the migration time ratio of Na equally when using both reference peaks: 1.06% for Na/'Cs' and 0.98% for Na/'Li'. This differs from "Sample IS" where using the Na/Li migration time ratio was clearly superior. When adjusting the migration time of Na using the relative distance between both reference peaks ("Na (new)" in Table 4.3-2; see Eq.3.3-1 in Chapter 3), the RSD was further improved to 0.71%, as illustrated in Figure 4.3-4 (before and after).

Figure 4.3-4. One electropherogram taken from each chip's analysis of 6mM NaCl. In (a) time scales are unadjusted, while in (b) peaks are shifted based on the analyte's relative distance to the BGE reference peaks. Electropherograms are drawn post-processing, and the baseline over/undershoots to the right of peaks are inherent to the raw data.

Table 4.3-2. Summary of migration time RSDs for all experiments.

	Na	'Cs'	'Li'	Na/'Cs'	Na/'Li'	Na (new)
Chip 1-3	1.30%	1.45%	1.65%	0.84%	0.72%	0.77%
Chip 1-9	0.97%	1.37%	1.47%	0.81%	0.76%	0.80%
Chip 3-2	1.30%	0.50%	1.04%	0.97%	0.33%	0.57%
Chip 3-3	1.11%	0.55%	0.92%	0.91%	0.37%	0.55%
Chip 4-1	1.16%	0.57%	0.95%	0.86%	0.33%	0.54%
Chip 4-7	1.45%	0.71%	1.25%	1.00%	0.37%	0.58%
All	2.93%	2.34%	3.69%	1.06%	0.98%	0.71%
Average	8.75 s	6.57 s	11.0 s	--	--	8.82 s
Sample IS	3.60%	2.33%	4.07%	1.76%	0.82%	0.57%
Average	8.76 s	6.41 s	10.6 s	--	--	8.59 s

4.3.4 | comparing measurements and simulations: calibration curves

Since the peak areas of 'Cs' and 'Li' vary with Na concentration differences (as seen previously in Figure 4a), it is useful to understand which regression type we need to apply to properly fit our calibration curves. To investigate this, we simulated electrokinetic-injection and separation steps for multiple NaCl sample concentrations using Simul 5. Figure 4.3-5a plots the NaCl concentration in sample well versus the simulated Na ion concentration in the injection channel after sufficient time has passed to allow for all sample ions to cross the stationary boundary. The simulations suggest that between 1-20mM, the EKI results in a slightly quadratic curve, which is then reflected in the area of the analyte peaks.

Figures 4.3-5b plots the separated peak areas taken from Simul 5 and compares it with the data collected from all microfluidic chips. Although the injected concentration of Na has a slightly quadratic dependence on the Na concentration in the sample well, the separated peak area can be approximated with high confidence as a linear line between 1-10mM Na concentration. The peak heights cannot, however; Figure 4.3-5c similarly plots theoretical to experimental values for separate peak heights between 1-15mM. For both plots, the theoretical calibration lines have only been scaled by a fixed constant to match the arbitrary values on the y-axis, and are otherwise not manipulated. Thus, theoretical curve shapes are in excellent agreement with the experimental results.

Figure 4.3-5. (a) Simulations of the NaCl concentration in the injection channel versus the Na ion concentration in the sample well after sufficient time has passed to allow for all sample ions to cross the stationary boundary. (b) Measurements and simulations of the Na peak area versus the Na ion concentration in the sample well, showing an approximately linear relationship between both and an excellent prediction. (c) Measurements and simulations of the Na peak height versus the Na ion concentration in the sample well, showing a quadratic relationship between both and an excellent prediction.

Calibration curves for the five measured concentrations of NaCl were built for both peak areas and heights, by performing a standard polynomial regression of order 2. All chips gave good intra-chip correlation coefficients (R^2) of approximately 0.99, aside from one (Chip 4-1). Table 4.3-3 includes intra-chip coefficients for both Na peak area and height, along with a linear regression of the Na peak areas, further demonstrating the ability to use a linear approximation instead. Each intra-chip line is further plotted in Figures 4.3-6a and 4.3-7a.

Chip-to-chip correlation coefficients for Na improved from the Sample ISTD experiment set: 0.9838 for peak area and 0.9695 for peak height. When 'Li' is used as a reference standard, these values improve to 0.9965 and 0.9882 for area and height, respectively. In the Na/'Cs' plot, however, one curve (Chip 3-2) is distinctly different from the other chips at all concentrations, resulting in an unimproved correlation coefficient of 0.9792. If this chip's data is removed for all curves, the others regressions remain relatively unchanged – except for Na/'Cs' peak area, which improves to 0.9934. It is unknown why this chip performs significantly different than the others. When considering Na/'Cs' heights, the correlation coefficient is well-matched with Na/'Li' at 0.9858.

Table 4.3-3. Summary of regression fitting for the Na calibration curves.

	Peak Area				Peak Height		
	$R^2_{Na(x)}$	$R^2_{Na(x^2)}$	$R^2_{Na/Cs}$	$R^2_{Na/Li}$	$R^2_{Na(x^2)}$	$R^2_{Na/Cs}$	$R^2_{Na/Li}$
Chip 1-3	0.984	0.991	0.996	0.998	0.990	0.992	0.997
Chip 1-9	0.991	0.996	0.997	0.998	0.992	0.994	0.998
Chip 3-2	0.994	0.994	0.996	0.998	0.990	0.993	0.997
Chip 3-3	0.995	0.995	0.997	0.998	0.990	0.995	0.997
Chip 4-1	0.964	0.965	0.998	0.998	0.963	0.997	0.998
Chip 4-7	0.990	0.993	0.996	0.998	0.995	0.996	0.999
All	0.984	0.984	0.979 (0.993)	0.997	0.970	0.986	0.988
Sample IS	0.943	--	0.991	0.971	--	--	--

Figure 4.3-6. Intra-chip calibration curves: (a) Na peak areas only, (b) Na/'Cs' PAR, and (c) Na/'Li' PAR. Each error bar denotes the standard deviation of six measurements from one chip. Chip-to-chip correlation coefficients are given in Table 4.3-3. The lines show a standard polynomial regression of order 2 through the data points.

Figure 4.3-7. Intra-chip calibration curves: (a) Na peak heights only, (b) Na/'Cs' height ratio, and (c) Na/'Li' height ratio. Each error bar denotes the standard deviation of six measurements from one chip. Chip-to-chip correlation coefficients are given in Table 4.3-3.

4.3.5 | reproducibility of peak area

General considerations

Figure 4.3-8a compares peak area RSDs at each NaCl concentration, for each microfluidic chip and all chips. Unlike in the "Sample IS" experiment set, there is no trend of smaller NaCl sample concentrations having larger variability; all chip-to-chip RSDs are ~5-7%. Outlier concentrations with large RSDs (7-10%) are random, ruling out specific chips, batches, experiment days, and/or NaCl concentrations. Chip-to-chip variability is approximately 1.5-2 times the intra-chip variability – much better than in the "Sample IS" experimental set reported in chapter 3 with a 3-4 times increase from intra-chip to chip-to-chip RSDs. Figure 4.3-8b further compares peak area and height RSDs for this experiment set with the results from "Sample IS"; at low concentrations (2mM, 4mM) a 2-fold increase in precision is seen. Also of note is the similar RSDs for both Na peak areas and heights, with neither being clearly superior. We attribute these improved chip-to-chip variabilities *before applying the BGE Ref* to a reduction in analyte-glass interactions owing to different chip-to-chip surface chemistries, since a comparable amount of Cs and Li ions exist everywhere in the system. Values for Na peak area and height RSDs are given in Table 4.

Improvement with the BGE Ref

The ratios with both reference standard peaks ('Cs' and 'Li') improves the RSDs when using both peak areas and heights (Figures 4.3-8c-e). When considering peak area ratios, the 'Li' reference peak performs better as a standard than 'Cs' due to the significantly different values for Chip 3-4. If only the other five chips are considered, the PAR values are comparable to 'Li'. This contrasts the "Sample IS" results, where the Cs peak was clearly superior.

As a means to judge how well each reference standard performed, experimental correlation coefficients (ρ) for the Na peak with 'Cs' and 'Li' were calculated using Eq.5 in Chapter 3. These five coefficients (one for each NaCl concentration) were then averaged, and are given in Table 4. When Chip 3-4 is not considered for the Na/'Cs' PAR, the average correlation is almost identical for all ratios: 0.831 for Na/'Li' heights, 0.853 for Na/'Cs' areas, and 0.856 for Na/'Cs' heights and Na/'Li' areas. This demonstrates a much stronger correlation between the peaks than was seen for Na and Li in the "Sample IS" experiment set at 0.636; however, Na and 'Cs' correlation is still superior at 0.922.

Figure 4.3-8. Relative standard deviations (RSDs) for each concentration of Na under various conditions. In (a), the intra-chip peak area RSDs are shown next to chip-to-chip RSDs, which are approximately 1.5 times larger. In (b), peak area and height RSDs are compared to those from "Sample IS" (no standards in the BGE); the Na RSDs values are noticeably better, especially at lower concentrations.

Figure 4.3-8. Relative standard deviations (RSDs) for each concentration of Na under various conditions. In (c), peak area RSDs and PAR are compared. In (d), peak height RSDs and height ratios are compared.

Figure 4.3-8: Relative standard deviations (RSDs) for each concentration of Na under various conditions. In (e), all RSDs for this experiment set are compared. In (f), the 'Cs' ratios are compared with values from the "Sample IS" experiment set.

Table 4.3-4. Summary of chip-to-chip RSDs for all peak areas, heights and ratios, with the averaged correlation coefficient for all Na concentrations (calculated using Eq.3.3-2 from Chapter 3).

	Peak Height RSD [%]			Peak Area RSD [%]			
	Na	Na/'Cs'	Na/'Li'	Na	Na/'Cs'	Na/'Cs' (no 3-2)	Na/'Li'
2mM	7.31	3.68	3.23	6.81	5.17	4.40	4.71
4mM	5.83	3.33	2.71	5.67	5.18	3.28	3.54
6mM	4.92	2.98	3.32	5.35	5.01	2.43	3.71
8mM	5.10	2.89	4.04	6.07	4.79	3.07	2.69
10mM	7.01	3.20	4.80	5.01	5.04	2.17	2.54
Correlation ρ	--	0.856	0.831	--	0.729	0.853	0.856
Sample IS, ρ	--	--	--	--	0.922	--	0.636

While an indication of how well the reference standard performs, the final precision itself is more important than the correlation. Figure 4.3-8f compares the RSD values for the Na peak areas and heights without any reference standards in the BGE, with the Na/'Cs' ratios, and the Na/Cs PAR from "Sample IS". We can conclude that the Na/'Cs' peak height ratios are clearly superior to the Na/Cs PAR from "Sample IS", as well as the Na/'Cs' PARs without Chip 3-4.

4.4 | Conclusion

The "BGE Ref" method is a promising means for correcting imprecision in chip-to-chip variation, due to its apparent insensitivity to reference standard substance ('Cs' and 'Li' performed equally well) and the improvement in the Na peak precision even without using any ratios with reference peaks. Under these experimental conditions, this method is even superior to the traditional method.

4.5 | References

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CHAPTER 5

improved quantification for point-of-care capillary electrophoresis by incorporating an internal standard in the BGE – ii. sample matrix with controlled composition^{*}

5.1 | Introduction

In the last decade, microchip capillary electrophoresis has been developed into a platform for point-of-care diagnosis of small ions, while commercialization is under way.¹⁻⁴ Addition of ISTD to the sample is a standard technique to improve run-to-run quantitation in conventional CE⁵⁻⁷ and MCE^{8,9}. In chapter 3 of this thesis, we demonstrated that adding an ISTD to the sample not only can be used to improve run-to-run quantitation, but also to improve chip-to-chip precision, as needed when disposable microchips are used.¹⁰ However, in POC analysis, addition of ISTD to the sample would require an extra sample preparation step performed by either an unskilled operator or the device itself. The last chapter demonstrated how standards added to the background electrolyte can give rise to a viable reference peak for improving the precision of microchip capillary electrophoresis measurements in a simple system. However, as was shown in Section 4.1, the reference peak sizes are strongly dependent on both the BGE matrix and the sample matrix. As the conductivity of the sample solution changed (by varying the concentration of the analyte),

^{*} paper in preparation.

the amplitudes and areas of the reference peaks changed as well. While we demonstrated that this did not eliminate the possibility of using the BGE-added standards as a correction factor for the measurement, the question naturally arises how the method will perform with a solution of less-variable composition.

Ionic concentrations in our blood are strictly controlled within a few mmol/L, and with the large amount of sodium present compared to other ions (3-4 times; see Table 5.1-1), the conductivity is much less variable than other samples.

Table 5.1-1. Typical blood plasma concentrations for abundant inorganic cations.¹¹

	"Normal" Blood Plasma Range
Na⁺	135 – 145 mmol/L
K⁺	3.5 – 5.0 mmol/L
Ca²⁺	2.2 – 2.65 mmol/L
Mg²⁺	0.6 – 1.1 mmol/L

This means that even with major fluctuations in the other ions (potassium, calcium, magnesium), the size of the BGE-added reference peaks should vary much less than in the previous chapter and that their magnitude should be primarily dependent on the relatively constant sodium concentration. Thus, the behaviour of the BGE-added standard may perform closer to that of a sample-added internal standard under these conditions.

To investigate this, we explore the effect of a BGE-added CsCl reference system peak on the chip-to-chip reproducibility of K⁺ quantification, using a simple solution containing cation concentrations similar to the normal ranges in blood plasma. The BGE constituents and behaviour of the BGE-added reference peaks were partly planned on the basis of predictions by the simulation program, Simul 5. As in previous chapters, we aimed to repeatedly measure multiple potassium concentrations across six different microchips. Due to microchip thin-film electrode damage incurred during setup modification prior to this experiment set, measurements were only able to be performed on four chips. This allowed us to draw conclusions from six measured K⁺ concentrations, measured thirty times at each concentration (six sample loads of five runs each), on four microchips: approximately 720 electropherograms. We further compare the performance of the 'Cs' reference peak to the performance of the Na sample peak, which has been used as a pseudo-internal standard due to its approximately constant concentration in blood samples by the company Medimate⁴.

It should be noted that this investigation also has a clinically-related application. Hyperkalemia is a medical condition where too many potassium ions are present in the blood, and is seen frequently in hospital Emergency departments. Typically diagnosed from blood serum samples, moderate hyperkalemia is defined as a potassium ion concentration of above 6 mmol/L, with severe hyperkalemia defined as above 7.0 mmol/L. Outward symptoms are typically not present until the hyperkalemia is severe, at which point the cardiovascular system can become compromised, which can subsequently lead to death. Thus, a device which can quickly diagnose this condition at the patient's bedside in the Emergency room, with precise quantification, would be very beneficial. In this context the research as presented in this chapter was performed.

5.2 | Materials and Methods

5.2.1 | MCE system

This experiment set used the same chip holders, experimental procedure and setup as in previous work (see Chapter 3.2), with some modifications.

The high voltage system was replaced with an instrument capable of operating at higher voltages and collecting the current information (HVS448 3000-LC, LabSmith Inc., Livermore CA, USA), which allowed us to increase the voltage to perform faster separation steps. The pinched injection protocol remained the same as in previous work: 1000V applied to the sample inlet and BGE outlet, 800V to the BGE inlet, and 0V to the sample waste reservoir. Separation then applied 1500V to the BGE inlet, 975V to the sample inlet, 950V to the waste reservoir, and 0V to the BGE outlet. Higher separation voltages would sometimes result in the formation of a gas bubble between the thin-film conductivity electrodes due to the electrolysis of water, invalidating the measurement. Injection and separation times were 45s and 35s, respectively, for all electrophoretic runs.

Four chips were chosen from the same initial three batches (wafers) fabricated for the first experiment set, of which only one chip was used in previous experiments (3-2). All chips were cleaned by flowing 0.1N NaOH and water through the channels as in previous chapters, and then were left to acclimatise to the BGE buffer constituents (sans reference standard) for at least 90 minutes. Microchips were then filled with, and stored in, Milli-Q water.

5.2.2 | reagents

BGE solutions were mixed daily from stock solutions and consisted of 120mM MES-His, 0.01% (w/v) HPMC, 250mM 18-crown-6 (to effectively resolve the potassium peak from

the cesium system peak), 5mM of NaCl (to improve the analyte peak shape), and 1mM of the CsCl reference standard. Details on the design choices for this BGE solution are given in Section 5.3.1. Sample solutions were also prepared from stock daily and contained 140mM NaCl, 0.01% HPMC, 2mM CaCl_2 , 1mM MgCl_2 , and one of six different concentrations of KCl (3, 4, 5, 6, 7 and 8mM).

5.2.3 | baseline-fit

As in the previous chapter, the background correction method developed by V. Mazet, et al.¹² was used (as found on MATLAB's File Exchange). However, the polynomial fit was reduced from a 5th order function to a 3rd order, due to the significant influence of the very large sodium peak. Figure 5.2-1 demonstrates how the increased order caused the baseline fit to curve near the sodium peak, resulting in the 'Cs' peak height/area increasing and the K peak decreasing. The 3rd order function was judged to do a better job of approximating the baseline, although it still does not perfectly fit the signal leading up to the 'Cs' peak.

Figure 5.2-1. Two baseline fits to the same electropherogram, both using the background correction method from MATLAB's File Exchange. The 3rd order polynomial fit ($p=3$), with a symmetric Huber function and threshold of 0.01, approximates the baseline better than the 5th order polynomial used in Chapter 4.

Peak areas are again estimated using the full width at half maximum, with the corresponding migration time taken as the half maximum time on the leading edge. Peak height times are taken as the maximum/minimum point.

5.2.4 | simul 5

All simulations performed in this chapter are done using the freeware program Simul 5¹³. Using mathematical models, the software simulates a spatial image of the electromigration and diffusion of electrolytes in free solution, while preserving mass conservation, acid-base equilibria, and electroneutrality laws. It provides electrophoresis researchers with a platform to inspect system peaks, sample (de)stacking, and help optimise experimental conditions.

However, since it does only model a single capillary, to fully understand a microchip injection-separation pair, a two-step simulation must be performed. First, the injection step is modelled by breaking the capillary into two segments, with the sample in one segment and the BGE in the other. The sample is then electrokinetically injected into the BGE zone, and the program is left to run until a very distinct plateau region appears for all constituents entering the BGE zone of the capillary. Choosing one position in the plateau region, the concentration of each constituent was recorded, and subsequently used for the injected sample concentration in the separation step.

In the simulations performed here, ionic strength was not accounted for. This is due to the heavy computing stress these calculations require, and to negative value errors resulting from spurious oscillations formed at sharp boundaries. Thus, the Ca and Mg peaks should be disregarded in all simulated electropherograms. These peaks have slower migration times under these conditions due to their sensitivity to ionic strength, and appear later in the experimental electropherograms (hidden within the Na peak).

5.3 | Results and Discussion

5.3.1 | experiments I: increasing the NaCl sample concentration

In Figure 4.4 of the previous chapter, we saw that as the Na concentration increased in the sample, the Cs and Li reference standard peaks increased and decreased in magnitude, respectively. To further investigate how the Na concentration influenced these peaks, its concentration in the sample was increased 12-fold. Figure 5.3-1 plots electropherograms taken from the same chip with a BGE of 100mM MES-His, 0.01% HPMC, 0.25mM KCl, and 7.5mM of CsCl and LiCl (BGE-added reference peaks). The sample consisted of 100mM MES-His, 0.01% HPMC and one of six concentrations of NaCl (6, 10,

20, 40, 80, and 120 mM). We can see that as the Na sample concentration increases, the 'Cs' peak does as well, and becomes asymmetrical and broad like the Na peak. The 'Li' peak continues to decrease in magnitude, until at about 40mM, where the peak just begins to become positive. In terms of migration times, the 'Cs' peak remains fixed in the same position; however, the 'Li' peak arrives later and later, as the width of the Na concentration increases in size. Thus, the amount of BGE-added reference standard needed to be reconsidered.

Figure 5.3-1. Experiments showing how the 'Cs' and 'Li' BGE reference peaks change in amplitude and area as the sodium concentration is increased in the sample. BGE: 100mM MES-His, 0.01% HPMC, 0.25mM KCl, and 7.5mM of CsCl and LiCl. Sample: 100mM MES-His, 0.01% HPMC, and varied NaCl concentration as shown on the figure's legend.

5.3.2 | simulations I: modifying the BGE composition

Adding NaCl to the BGE

While the large amount of sodium ions present in the sample keeps its composition relatively constant, it also results in a large asymmetrical peak which is broadened by electrodispersion. As seen earlier, this makes the baseline more difficult to estimate, particularly if the analyte is in close proximity to the Na peak, and can lead to dispersions of other peaks in the electropherogram. In attempts to reduce the magnitude of this peak, we used Simul 5 to investigate the effects of adding a relatively small amount of NaCl to the BGE. The samples simulated in Figure 5.3-2 all contained 140mM NaCl, 2.5mM CaCl_2 , and 1.5mM MgCl_2 . The BGE was composed of 100mM MES-His, 2mM CsCl, and either 0, 10, or 20mM of NaCl.

The addition of NaCl to the BGE has an interesting effect on the Na peak: rather than reducing its size, it instead reduces the peak's effective mobility. The magnitude of the peak increases, presumably due to sample stacking of all analyte components due to the BGE's increased conductivity, but the peak appears later. While the Ca and Mg peaks are not accurate representations due to the ionic strength not being factored into the mathematical model, it can be seen that reducing the effective mobility of the broad Na peak results in better peak shapes for both the analytes which migrate faster than the Na zone and the 'Cs' peak. We speculate that this is due to these analyte zones "escaping" the Na moving analyte zone quicker, and thus being subjected to less electrodispersive effects.

Overlap of K and 'Cs' Peaks

Figure 5.3-3 plots three simulated electropherograms with either 5mM KCl in the sample, 2mM CsCl in the BGE (added reference standard), or 5mM CsCl in the sample, to demonstrate the overlap of these peaks if they were present in the same electropherogram. Since K is our analyte of interest, the effective mobility needs to be reduced to resolve it from the Cs reference peak. One common way of modifying the mobility of potassium is through the addition of 18-crown-6¹⁴. Ether additives cannot be modelled easily using Simul 5, however, so we manually adjusted the peak's mobility from 76.2 to 68.2 ($10^{-9} \text{ m}^2/\text{V}\cdot\text{s}$) for the remainder of the simulations.

Li as a BGE-Added Reference Peak

When used as a BGE-added reference peak in the previous chapter, Li performed nearly as well as the Cs peak; thus, we also investigated adding LiCl to the BGE using Simul 5. Figure 5.3-1 previously showed that a large amount of Na in the sample results in the 'Li' peak

Figure 5.3-2. Simulations determining how adding NaCl to the BGE affects the size and shape of the Na analyte peak. Rather than reducing the peak size, the sodium peak's effective mobility was reduced and the peak increased in size (due to sample stacking). The sample solution in all cases contained 140 mM NaCl.

Figure 5.3-3. Three superimposed simulations demonstrate the overlap of the Cs system peak and K analyte peak, requiring a BGE additive to modify the effective mobility of the K analyte peak so that it is resolvable. Sample otherwise contains 140mM NaCl, 2.5mM CaCl₂, and 1.5mM MgCl₂. BGE consists of 100mM MES-His and 20mM NaCl.

Figure 5-3-4. (a) Simulations demonstrating the effect of adding Li as a BGE-added reference standard when 20mM NaCl is also present in the BGE. Addition of Li is seen to split the Na peak, while not generating a useful system peak. This is further illustrated in (b), which plots the change in BGE constituents of the 2mM LiCl simulation. Thus, only CsCl is used as a BGE reference standard in the experiment set reported in this chapter.

becoming a positive system peak. When 20mM NaCl is also present in the BGE, the addition of even small amounts of LiCl causes the Na peak to split into two peaks (Figure 5.3-4a). The more LiCl that is added to the BGE, the more these two peaks separate from each other, with the first having a higher effective mobility and the second, lower.

Figure 5.3-4b shows the change in BGE constituent concentrations when 2mM of LiCl is added to the BGE. The 'Cs' reference peak is still clearly visible as the most depleted zone of Cs ions; however, it is not clear which of the latter peaks is "Na" and which is 'Li'. This means that LiCl cannot be used as a BGE-added reference standard in this work, as the generated system peak is no longer useful.

5.3-3 | simulations II: varying experimental conditions

A true internal standard is a substance which is different from the analyte of interest, but which is influenced by other experimental and sample preparation conditions in the same way. In the previous chapter, we saw that the magnitude of the BGE-added system peaks depended on the amount of sample which was injected as desired; however, they also were dependent on the sample composition and conductivity. Here, we proposed that the large amount of sodium in the sample (relative to the other constituents) would make the latter effect less prominent, resulting in a reference standard closer to the ideal.

To investigate this in a model world, we used Simul 5 to vary the amount of KCl in the sample between 2.5mM and 7.5mM. Figure 5.3-5a plots 3 concentrations of KCl in the sample, which otherwise contains 2.5mM CaCl_2 , 1.5mM MgCl_2 , and 140mM NaCl. The BGE is as designed in previous sections (100mM MES-His, 0.01% HPMC, 20mM NaCl, 2mM CsCl). In proportion to the large amount of NaCl, the varied KCl concentration has minimal change on the sample matrix, resulting in the other peaks remaining primarily unchanged (as we would hope for an ideal standard). It can also be seen from Figure 5.3-5b that the heights and areas of the K peaks both should increase in a linear fashion. This is in contrast to the second-order polynomial fit described by simulations and experiments in Chapter 4.

On the other hand, if the injected sample plug size is varied (which in real life can occur due to chip-to-chip differences such as channel depth variations), we see from the simulations in Figure 5.3-6 that the reference standard does still change in magnitude, as desired. Thus, with a sample of relatively unchanging sample composition, we would expect the BGE-added reference peaks to perform better and closer to that of a true internal standard.

Figure 5.3-5. (a) Simulations illustrating that the 'Cs' reference peak does not change in size as the K peak is varied between 2.5mM and 7.5mM, as indeed hypothesized at the start of the experiments. **(b)** Plot of the K peak height and areas collected from the simulations. A linear regression was performed on the data, and correlation coefficients agree that the data for both height and area should be linear (unlike Chapter 4, which followed second-order polynomial fits).

Figure 5.3-6. Simulations illustrating that the 'Cs' reference peak does still change with variations in the sample plug size (e.g. due to chip-to-chip differences in channel depth), as we would expect for it to perform as a reference measurement peak.

5.3.4 | final BGE composition

The final BGE used for modelling in Simul 5 was 100mM MES-His, 20mM NaCl, and 2mM CsCl. However, these concentrations were not optimised for the best separation; rather, they were values used to illustrate how the electropherograms would conceptually change with certain BGE-added constituents. When we moved away from simulations to practical use, the concepts developed in the simulations were applied to a few short experiments used to determine which BGE composition appeared to give the best K and 'Cs' peak shapes (see Figure 5.3-7). This was found to be a BGE of 120mM MES-His, 0.01% HPMC, 5mM NaCl, 2.5mM 18-crown-6, and 1mM CsCl. It should thereby be noted that this BGE was not yet optimised for the best reproducibility, and the relative standard deviations given in this chapter will reflect this.

Figure 5.3-7. Comparison of different BGE systems tested with singular experiments to investigate K and 'Cs' peak shapes. (a) is a zoomed-in version of (b), where "MH" denotes the MES-His concentration and "crown" the 18-crown-6 concentration. Samples were all 140mM NaCl, 5mM KCl.

5.3.5 | experiments II: reproducibility of migration time

Intra-chip and chip-to-chip RSDs for peak migration times are given in Table 5.3-1. As expected, chip-to-chip migration time RSDs were larger than intra-chip values: approximately 4.0%, 3.5% and 3.5% for K, 'Cs' and Na, respectively, against intra-chip values of approximately 2%. The potassium peak is the most variable here, which differs from previous chapters where RSD increased with retention time. This is likely a result of the 18-crown-6 added to the BGE to adjust the potassium peak's effective mobility. While chip-to-chip RSDs are about the same as the most variable peak in previous chapters (Li), intra-chip RSDs are almost double, which could be indicative of the BGE and electrophoretic protocols requiring further optimisation.

Despite the increased variability in the analyte peak migration time, the BGE-added reference peak improves the RSD to 0.57% when the ratio of the peak height times is used. This value is as good as the best deviation obtained in Chapter 3 using the relative distance between both internal standards. In comparison, the Na peak as an internal standard only performs half as well, improving the RSD to 1.02%. Under these experimental conditions, the peak height migration times are more reliable when the ratio is taken, whereas in chapters 3 and 4 the half-height time on the leading edge of the peak had the best precision.

Table 5.3-1. Summary of migration time RSDs for all experiments, comparing the time taken at (t @) the peak's maximum and its half maximum on the rising edge.

	t @	K	'Cs'	Na	K/'Cs'	K/Na
Chip 3-2	max	1.65%	1.88%	1.31%	0.40%	0.37%
	½ max	1.66%	1.75%	1.28%	0.41%	0.24%
Chip 3-9	max	1.36%	1.63%	0.99%	0.42%	0.42%
	½ max	1.63%	1.55%	1.02%	1.36%	1.51%
Chip 4-6	max	3.01%	2.73%	2.51%	0.56%	0.53%
	½ max	2.74%	2.65%	2.47%	0.46%	0.45%
Chip 4-10	max	2.30%	2.45%	2.21%	0.21%	0.40%
	½ max	2.25%	2.49%	2.30%	0.75%	0.83%
All	max	4.01%	3.51%	3.57%	0.57%	1.02%
	½ max	4.09%	3.52%	3.46%	1.09%	1.31%

5.3.6 | experiments III: calibration curves

Linear regressions were performed to build calibration curves for the five measured concentrations of KCl, as described in Section 5.3.3. Correlation coefficients (R^2) are given for individual chips, along with a final chip-to-chip value, in Table 5.3-2. The calibration curves themselves are plotted with error bars of one standard deviation in Figure 5.3-8a and 5.3-9a for potassium peak areas and heights, respectively. When peak heights are used, all individual chips fit the calibrations curves with coefficients of 0.97 or better. When peak areas are used, only Chip 3-2 performs well. This may very well be a result of poor baseline fitting (see Figure 5.2-1), resulting in poor standard deviations – particularly when measuring 3mM of KCl. It can be seen that the calibration curves differ strongly between chips, with varying slopes and offsets.

As mentioned, with our system we can investigate both the performance use of the BGE-added Cs and the Na present at constant concentration in the sample as an ISTD. Using the peak height and peak area ratios of potassium with the 'Cs' reference peak and the Na peak, do not consistently improve the correlation coefficient for each individual chip. It is only when all data is included (hence chip-to-chip variations considered) that the ratios improve the correlation coefficient. If peak areas are used, the peak area ratio of K/Na improves the correlation to approximately 0.85, whereas the K/'Cs' PAR has little effect. When peak heights are used, the K/'Cs' ratio performs even better than the K/Na PAR, improving the correlation to 0.93 from 0.70. Meanwhile, K/Na peak height ratio has little effect. It is at present unknown why the BGE-added reference peak performs better when heights are concerned, whereas it performs so poorly for areas; however, the authors suspect the baseline fitting method, as discussed in Figure 5.2-1. If this is correct, it stresses the huge importance of finding an optimal baseline-fitting procedure in systems like the present one.

Table 5.3-2. Summary of linear regression fitting for the K calibration curves.

	Peak Height			Peak Area		
	R^2_K	$R^2_{K/Cs}$	$R^2_{K/Na}$	R^2_K	$R^2_{K/Cs}$	$R^2_{K/Na}$
Chip 3-2	0.991	0.991	0.991	0.977	0.987	0.990
Chip 3-9	0.990	0.990	0.990	0.857	0.876	0.890
Chip 4-6	0.976	0.981	0.993	0.874	0.907	0.925
Chip 4-10	0.987	0.985	0.984	0.916	0.910	0.950
All	0.703	0.932	0.725	0.762	0.780	0.843

Figure 5.3-8. Intra-chip calibration curves: (a) K peak areas only, (b) K'/Cs' PAR, and (c) K/Na PAR. Each error bar denotes the standard deviation of six measurements from one chip. Chip-to-chip correlation coefficients are given in Table 5.3-2. The lines show a standard linear regression through the data points.

Figure 5.3-9. Intra-chip calibration curves: (a) K peak heights only, (b) K'/Cs' PHR, and (c) K/Na PHR. Each error bar denotes the standard deviation of six measurements from one chip. Chip-to-chip correlation coefficients are given in Table 5.3-2. The lines show a standard linear regression through the data points.

5.3.7 | experiments IV: reproducibility of peak area

General considerations

Figures 5.3-8a and b compare peak height and area RSDs, respectively, at each KCl concentration on each microfluidic chip, as well as for all chips. Intra-chip peak heights are the least variable, all chips and concentrations giving less than approximately 5% deviation. Chip-to-chip height variation, however, is 5x larger at the lowest concentrations (3, 4mM) and 4x larger at all other concentrations (5, 6, 7, 8mM) than intra-chip variation. Intra-chip RSDs for peak area are about 5-7%, with two clear outliers: 3mM of KCl on chips 3-9 and 4-10. This dramatic increase in RSD of the peak area at low concentrations may be due to the small K peak (near the system's limit of detection), which requires a more robust baseline-fit and peak area estimation than the one used. Chip-to-chip peak *area* RSDs are only 2-3x larger than intra-chip RSDs, aside from the 3mM concentration. Thus, chip-to-chip areas perform the best when considering only K peaks. Actual RSD are also given in Table 5.3-3.

Table 5.3-3. Summary of chip-to-chip RSDs for all peak areas, heights and ratios, with the averaged correlation coefficient for all K concentrations (calculated using Eq.3.3-2 from Chapter 3).

	Peak Height RSD [%]			Peak Area RSD [%]			
	K	K/'Cs'	K/Na	K	K/'Cs'	K/Na	Na
3mM	26.7	16.1	25.4	29.5	27.1	28.2	4.96
4mM	25.7	14.5	24.5	16.2	17.9	14.9	5.19
5mM	20.6	11.0	20.5	13.0	17.8	12.3	4.16
6mM	20.6	8.48	20.4	12.3	12.6	10.4	4.60
7mM	20.0	7.28	18.7	12.8	13.9	10.6	3.81
8mM	19.5	6.51	19.1	11.1	9.4	9.3	3.23
Correlation ρ	--	0.904	0.273	--	0.322	0.492	--

Improvement with the BGE Ref and Na ISTD

As seen with the calibration curves, the K/'Cs' peak area ratio has little-to-no improvement on the values for K areas alone (visualised in Figure 5.3-9c-e and Table 5.3-3). However, when heights are considered, the K/'Cs' peak height ratio improves the RSD by 2-3x at each KCl concentration. These RSD values are also better than the K peak areas, giving the most reproducible results. It is unknown why the peak height ratio performs much better than the peak area ratio, but may be a result of a baseline fit which doesn't match all the data well.

Figure 5.3-9. Relative standard deviations (RSDs) for each concentration of KCl under various conditions. In (a) and (b), the intra-chip peak height and area RSDs, respectively, are shown next to chip-to-chip RSDs.

Figure 5.3-9. Relative standard deviations (RSDs) for each concentration of KCl under various conditions. In (c), chip-to-chip peak area and height RSDs are compared to K/Cs' and K/Na ratios; the K/Cs' height ratio and K/Na area ratio perform the best. The best RSD values are then compared again in (d).

Figure 5.3-9. Relative standard deviations (RSDs) for each concentration of KCl under various conditions. (e) plots the peak area ratios with time-corrected height ratios, showing 1-2% improvement at each concentration. This is also reflected in (f) for time-corrected heights, which is not fully understood.

When the Na peak is used as an IS, the K peak heights and areas are only improved by about 1-2%. While the K/Na PAR performs better than the K/'Cs' PAR, this is primarily because the K areas themselves are more reproducible, rather than because Na is a good internal standard. This can be seen when considering the correlation co-efficient, calculated as previously given in Equation 3.3-2 and provided in Table 5.3-3. Na's peak area gives a correlation coefficient of 0.492 with the K peak area – about half of the coefficient for K and 'Cs' peak height, which shows superior correlation at 0.904. This poor correlation may be due to the fact that the Na peak is much larger than the K peak, and has a very different shape. Table 5.3-3 shows that the RSDs for the Na peak alone do not have much variation; thus, the baseline-fitting method here is not very suspect.

Figures 5.8-9e and f also plot ratios that have been corrected with the arrival time of the BGE-added reference peak or Na peak. For all ratios, both peak heights and areas, RSDs improve by an absolute 1-2%. Correction by dividing through the arrival time was not the only method investigated. Other investigated methods include: time-correcting both the K peak and the 'Cs'/Na peak; using the K heights and 'Cs' areas, and vice versa; and using both K and Na peak height/area to correct the K peak height/area. Of all of these, only the time-corrected reference standard method reliably improved the results, and thus, does not seem to be merely based on chance. This phenomenon is not well understood, and requires further investigation in view of its apparent reliable improvement.

5.4 | Conclusion

We conclude that the BGE-added reference standard method can be extended to samples with more constant compositions, and reliably improve the quantitation without resulting in a non-linear calibration curve when peak height ratios are used. Peak areas are usually the preferred method for quantitation – despite often being less precise – due to their more linear nature¹⁵. However, as shown in sections 5.3.3 and 5.3.6, the peak heights in this chapter demonstrate good linearity. While the RSD values found here are large and not yet useful, the authors suggest that further BGE and electrophoretic protocol optimisation, baseline-fitting investigations, and a larger sample set (i.e. 6 chips rather than 4) would also provide further improvement. Furthermore it must be remarked that the most successful correction procedures did result in reducing the chip-to-chip RSDs approximately two-fold at all concentrations.

5.5 | References

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CHAPTER 6

main conclusions and thoughts on further research

6.1 | Conclusions

A point-of-care platform for ionic analysis by microchip capillary electrophoresis (MCE) using a handheld reader and disposable microchips has recently been developed by the University of Twente start-up, Medimate. The aim of the research that is described in this thesis was to investigate the use of an internal standard in this platform or in comparable platforms. The research performed with this aim has been grouped in four chapters.

In **chapter two**, general background knowledge was provided that is needed for reading this thesis. Chapter two thus mainly concerns the electrokinetic, chemical, and detection aspects of MCE with conductivity detection. The chapter ends with an inventory of the different sources of analyte determination error in MCE and the use of internal standards to correct for those.

Internal standards (ISTDs) have been widely applied in capillary electrophoresis to improve the precision of the analyte quantification. However, in the reported analyses the same column is used over and over, i.e. the sources of error do not include variations in the column dimensions or the detector properties or dimensions. On the other hand, disposable microchips are used in MCE which do show such variations. The research question for **chapter three** therefore was to establish whether the use of an internal standard can also help to improve analyte quantification in case of single-use microchips.

In this chapter, we thus considered the improvement in *chip-to-chip precision* instead of the intra-chip precision by the addition of an ISTD. The chapter demonstrates that the chip-to-chip precision indeed improves by approximately a factor of two with ISTD addition, using the peak area ratio (PAR) of the internal standard and analyte as a tool. In these experimental conditions, the analyte was sodium, and both lithium and cesium were investigated as ISTDs. The chapter shows that an ISTD needs to be chosen with care, as lithium and cesium do not perform equally well. It also shows that either peak height and peak area can be used with varying results, but generally better results for the peak area. The conclusion of chapter three thus was that use of a properly chosen ISTD can indeed improve quantification in MCE with disposable microchips.

In chapter three, the internal standard was added to the sample in a known quantity prior to analysis, by the skilled laboratory worker. Such a procedure is also standard in capillary electrophoresis. In point-of-care applications however, such a dependence on operator skills is highly undesirable and some automated manner of ISTD addition therefore had to be invented. Inspection of the effect of adding small quantities of potassium to the background electrolyte (BGE) to improve peak shape in chapter three had shown the formation of a system peak at higher potassium concentrations. This led to the hypothesis that an ISTD – an ionic species not present in the sample – could be added to the BGE and that the magnitude of the system peak created by the presence of this BGE-ISTD could then be used to improve analyte quantification. This procedure would have the substantial advantage that not only additional sample preparation steps would be avoided, but also that possible additional on-chip modifications such as the addition of reservoirs with internal standards, and/or mixing and dissolution steps (which would inevitably be associated with additional error) would also be avoided.

The research question of **chapter four** thus was whether a BGE-ISTD could be used to improve quantification in MCE. Again, lithium and cesium were used as the ISTD ions while the sodium ion was the analyte. The PAR – this time of the 'Cs' and 'Li' system peaks and the Na analyte peak – again was used as improvement tool. The conclusion of this chapter was that indeed an improvement with approximately a factor of two was possible by this approach of BGE-added ISTD. An important observation that leads to some caution is that, although an increased precision is obtained when using the PAR, the calibration curves obtained between the analyte concentration and the PAR are not linear. This non-linearity is a consequence of the fact that the magnitude of the system peak is affected both by the ISTD concentration in the BGE and the properties of the sample, with the latter a function of the analyte concentration. Another factor that causes non-linearity is

electrokinetic stacking, which is the result of the electrokinetic injection that is used in the present device.

Some highly relevant sample matrices, such as blood, have a fairly constant composition. In this case the observed dependence on the sample characteristics is expected to be minimal, so that again a linear relationship between analyte concentration and PAR would be expected. This research question is investigated in [chapter five](#) of the thesis. In this chapter, it was found that the BGE-added ISTD can be used to improve analyte quantitation in the case of a fairly constant sample composition as well. Here, potassium was used as analyte, cesium as the BGE-added ISTD, and a high sodium sample concentration was used to keep the sample concentration fairly constant (mimicking blood). The determination of potassium in blood is clinically relevant to diagnose hyperkalemia, which can be life-threatening. Due to the constant sodium concentration in the sample, the performance of the BGE-added cesium standard could be compared to the performance of the sodium peak itself as an ISTD. As expected, approximately linear calibration curves were now obtained. Chip-to-chip precision could be improved with at least a factor of two by the use of the internal standard, with the potassium/'cesium' peak *height* ratio performing best as an ISTD and the potassium/sodium peak *area* ratio second best. In general, during the investigations performed in this chapter, the importance of a good algorithm for baseline correction was indicated, as improper baseline fitting can cause large errors in peak height and peak area determinations.

Two main conclusions of this thesis can now be drawn. The first conclusion is that ISTD addition can be used to improve chip-to-chip precision in MCE. The second conclusion is that – at least under certain circumstances - chip-to-chip precision can be improved by adding the ISTD to the BGE. The large advantage of this approach is that it is extremely simple and avoids introducing additional sources of error.

6.2 | Future Work

Future research following the work of this thesis can aim at several key points that have been identified. One is the sensitivity of the BGE-ISTD method to the sample characteristics, which can lead to a non-linear calibration curve. In general, a thorough theoretical basis for the BGE-ISTD method – for systems with electrokinetic injection – at present is lacking. In this thesis, this problem was solved by comparing the measurement results with the results from the Simul 5 program. However, an analytical theory of the system could be undertaken to aid future application of this procedure.

It is known that electrokinetic injection inevitably causes a non-linear calibration curve by its inherent bias, as was also found in this thesis. It thus is advisable to investigate hydrodynamic injection for point-of-care MCE, as it could be applicable for some sample categories.

A further point for future investigations is to find better algorithms for baseline correction. Baseline correction is of prime importance for peak height and peak area quantification. As the internal standard and analyte peaks inevitably appear at different times in the electropherogram, they will also inevitably always be affected differently by a baseline fitting program. In this thesis, many algorithms were investigated but none was found that performed optimally under all circumstances. Possibly every individual MCE system will be in need of its own dedicated software.

In chapter five, it was found that the RSD of potassium determination could be improved more by using the 'cesium' height/time ratio than the 'cesium' height. The same was found true for the sodium peak/time ratio. The theoretical background for this phenomenon merits further investigation.

The work presented in chapter five on using a BGE-added ISTD to improve the quantification of potassium is relevant for POC use in the emergency room of hospitals, where rapid and reliable data are needed to diagnose hyperkalemia. Here, experiments with clinical blood samples are the next step to take.

APPENDIX A

fabrication of capillary electrophoresis microchips

A.1 | Description

The chip consists of two Borofloat glass substrates bonded together. It is strongly recommended to always bond the smooth sides of the wafers (defined as the "front-side" in this document) together. To determine the front-side: hold the wafer with the big flat towards yourself, so the small flat is on the left; the upper side is the front-side.

As the top substrate (A) contains the powderblasted wells and we would like the option of running the chip without the mechanical reservoir extensions, the top plate will be of 1.1mm thickness and the bottom plate (B) of 0.5mm thickness. The wells will be blasted into the backside and the channels etched into the front-side. The bottom wafer will have tantalum/platinum electrodes buried into the front-side, so the electrode surface is in plane with the substrate surface. Tantalum is the adhesion layer for the platinum.

Figure A.1-1: Cross-section of the two glass substrates, with the front-side of each facing the other.

A.2 | Chip Design and Mask Layouts

Figure A.2-1: Chip design and the three masks used for fabrication. Top-right: Powderblasted wells. Bottom left: Etched microfluidic channels. Bottom-right: Recessed platinum electrodes and logos.

A.3 | Substrate A (Top Plate) – 1.1mm Borofloat Glass

Step	Process	Parameters	Notes
1	Standard Cleaning	2min 100% HNO ₃ I	<i>Removes any organic contaminants</i>
2	Wetbench 16	Quick Dump Rinse	
3	Standard Cleaning	10sec in 25% KOH @ 75°C dip	<i>Etches slightly to achieve a more reproducible surface</i>
4	Wetbench 17	Immerse in DI H ₂ O container	
5	Standard Cleaning Wetbench 16	10min ultrasonic in the first rinse water	<i>Removes any particles attached to the surface</i>
6		Quick Dump Rinse	
7		1min 100% HNO ₃ I	<i>Re-stabilises the glass surface.</i>
8		2min 100% HNO ₃ II	
9		Quick Dump Rinse	
10		Single wafer spin dryer	
11	Sputtering of Cr/Au layer	5min dehydration bake @ 120°C	<i>The Cr/Au layer acts as a mask during the HF etching of the channels. (HF diffuses through photoresist).</i>
12	Sputterke • use Ar flow to adjust pressure • Base pressure: < 1.0 e-6mbar	Cr Target • Deposition rate = 15 nm/min 2min sputtering for 30nm layer	
13	• Sputter pressure: 6.6 e-3mbar • power: 200W	Au Target • Deposition rate = 45-50 nm/min 3min sputtering for 150nm layer	
14	Photolithography Wetbench 21	Resist • Olin Oir 907-17 • 30sec, 4000rpm on spincoater	
15		90sec pre-bake @ 95°C	<i>Proceed immediately! If not, 5min dehydration bake @ 120°C</i>
16	Photolithography Electronic Vision Group EV620 Mask Aligner	• Hg-lamp: 12 mW/cm ² • Hard-contact • 5sec exposure time	
17	Photolithography Wetbench 21	Development • 30sec in Beaker 1 • 15-30sec in Beaker 2	<i>Proceed immediately between beakers and to next step!</i>
18		Quick Dump Rinse	
19		Single wafer spin dryer	

20	Inspecting Microscope	Ensure features are developed as desired without defects.	<i>If not, 2min 100% HNO₃ on WB 7, QDR, spin-dry, and return to step 14.</i>
21	Photolithography Wetbench 21	10min post-bake @ 120°C	
22	Lamination Dicing Room	Cover backside of wafer with blue dicing foil	<i>Protects against HF or the surface will become blurry.</i>
23		30sec bake @ 70°C (hotplate)	
24	De-scum Wetbench 1	5min UV ozone	<i>Removes scum from lithography and makes surface hydrophilic.</i>
25	Wet Etch Wetbench 7	Au Etchant (KI/I₂) ~1min, or until the metallic Cr colour appears (will first go dark/rough)	<i>Will first look dark due to rough gold patches on the chromium layer</i>
26		Cr Etchant ~20sec, or until the local parts become transparent. No longer!	<i>Chromium under etches quickly due to electrochemistry!</i>
27		Glass Etchant • 33% HF (fresh, 50%HF:H ₂ O 2:1 @ 19°C) • Etchrate: 1,9 µm/min (new) 3min 15sec for ~6µm	<i>Solution needs to sit in the wet bench for a few hours prior to use. Make sure to use plastic carrier instead of glass!</i>
28		Quick Dump Rinse	
29		Single wafer spin dryer	
30		Remove dicing foil, pulling parallel to surface, not perpendicular. Leave in bench to out-gas (~24hr).	<i>The foil absorbs HF, which is very dangerous.</i>
31	Measuring Veeco Dektak 8	Measure etch profile of channels and save for records.	<i>If channels are not deep enough, return to 22</i>
32	Cleaning	2min 100% HNO ₃	<i>Removes photoresist.</i>
33	Wetbench 7	Quick Dump Rinse	
34		Single wafer spin dryer	

35	Photolithography Wetbench 21	2min pre-bake @ 120°C	
36	Photolithography Electronic Vision Group EV620 Mask Aligner	Cut powderblast foil BF410, remove hard protection foil and roll out over wafer and place between two pieces of cleanroom paper.	
37		Laminate • pre-heated @ 105°C; speed 2	
38		Cut out wafer (flush) with a scalpel.	
39		Exposure • Hg-lamp: 12 mW/cm ² • Bottom-side • Crosshairs	• Alignment mark 2-2 • Soft-contact • 15sec exposure time
40		Place in single wafer box, covered with aluminum foil.	<i>Protects the photoresist.</i>
41	Lamination Dicing Room	Cover backside of wafer with blue dicing foil	<i>Protects against powderblasting or face will become blurry.</i>
42		30sec bake @ 70°C (hotplate)	
43		Peel off protection from blasting foil while wafer is still warm.	
44	Development BIOS CR2516	Spray 2% Na ₂ CO ₃ solution at ~40°C until developed parts lift-off.	
45		Rinse in DI water from tap	
46		Spin-dry for 30 sec	
47		Bake on 70°C hotplate for 90 sec	
48	Powderblasting CR 5th floor	<ul style="list-style-type: none"> • Grain size: 29 µm • Flow: minimal • Y-speed: 10mm/min (new SW); 2 (old SW) 	<ul style="list-style-type: none"> • X-step 0,7mm • Scans: 2 • Area: 65x82mm Cover Alignment Marks!!

49	Delamination CR2516	Wash wafer with warm DI water from the tap.	
50		Remove dicing foil, pulling parallel to surface, not perpendicular.	
51		~5min acetone bath or until the powderblast foil ripples off.	
52		Rinse with acetone and then immediately with IPA.	
53		10min ultrasonic DI water bath	<i>Removes powder particles.</i>
54		Spin-dry and place in clean box.	
55	Cleaning Wetbench 11	10min ultrasonic DI water bath	<i>Removes any particles attached to the surface.</i>
56		Quick Dump Rinse	
57		Single wafer spin dryer	
58		5min fuming HNO ₃	<i>Removes foil particles.</i>
59		Quick Dump Rinse	
60	Wet Etch Wetbench 7	Au Etchant (KI/I₂) ~1min, or until the metallic Cr colour appears (will first go dark/rough)	
61		Quick Dump Rinse	
62		Cr Etchant ~20sec, or until the local parts become transparent. No longer!	
63		Quick Dump Rinse	
64		Single wafer spin dryer	

A.4 | Substrate B (Bottom Plate) – 0.5mm Borofloat Glass

Step	Process	Parameters	Notes
1	Standard Cleaning	2min 100% HNO ₃ I	<i>Removes any organic contaminants</i>
2	Wetbench 16	Quick Dump Rinse	
3	Standard Cleaning Wetbench 17	10sec in 25% KOH @ 75°C dip	<i>Etches slightly to achieve a more reproducible surface</i>
4		Immerse in DI H ₂ O container	
5	Standard Cleaning Wetbench 16	10min ultrasonic in the first rinse water	<i>Removes any particles attached to the surface</i>
6		Quick Dump Rinse	
7		1min 100% HNO ₃ I	<i>Re-stabilises the glass surface.</i>
8		2min 100% HNO ₃ II	
9		Quick Dump Rinse	
10		Single wafer spin dryer	
11	Vapor priming Wetbench 28	<ul style="list-style-type: none"> • Furnace: Lab-line Duo-vac-oven • low pressure 5min dehydration bake @ 150°C	<i>If sat in box for a few days, 10min UV ozone prior to this step!</i>
12		5min HMDS prime @ 150°C	
13		Let wafer cool to room temperature.	
14	Photolithography Wetbench 21	Resist <ul style="list-style-type: none"> • Olin Oir 907-17 • 30sec, 4000rpm on spincoater 	
15		90sec pre-bake @ 95°C	
16	Photolithography Electronic Vision Group EV620 Mask Aligner	<ul style="list-style-type: none"> • Hg-lamp: 12 mW/cm² • Hard-contact • 5sec exposure time 	
17	Photolithography Wetbench 21	Development <ul style="list-style-type: none"> • 30sec in Beaker 1 • 15-30sec in Beaker 2 	<i>Proceed immediately between beakers and to next step!</i>
18		Quick Dump Rinse	
19		Single wafer spin dryer	

20	Inspecting Microscope	Ensure features are developed as desired without defects.	<i>If not, 2min 100% HNO₃ on WB 7, QDR, spin-dry, and return to step 11.</i>
21	Photolithography Wetbench 21	30min post-bake @ 120°C	
19	De-scum Wetbench 1	5min UV ozone	<i>Removes scum from lithography and makes surface hydrophilic.</i>
20	Wet Etch Wetbench 6	Glass Etchant • BHF (standard solution @ 19°C; needs to sit in wetbench for a few hours prior) • Etchrate: 24 nm/min 7min for 168nm	<i>Etch a recess in the glass to have the electrode in plane with surface after sputtering.</i>
21		Quick Dump Rinse	
22		Single wafer spin dryer	
22	Measuring Veeco Dektak 8	Measure depth of recess to verify sputter amounts	<i>Accuracy of ~7nm</i>
23	Pre-conditioning Wetbench 1	5min UV ozone	<i>Proceed immediately!</i>
24	Sputtering of Ta/Pt layer Sputterke • use Ar flow to adjust pressure	Ta Target • Deposition rate = 16,5 nm/min 1min 30sec for 25nm layer	<i>Sputter uniformity is ~10%; centre will be thicker than the edge. Preferred that centre be flush with surface rather than edge, to prevent bad "direct bonding".</i>
25	• Base pressure: < 1.0 e-6mbar • Sputter pressure: 6.6 e-3mbar • power: 200W	Pt Target • Deposition rate = 22-27 nm/min ~6min sputtering for ~143nm	
26	Lift-off Wetbench 11 (left) • Use dedicated metal beakers and carriers	Fill metal beaker with Acetone technical and immerse wafer. Ultrasonic for 1min and then let stand for a few hours.	<i>Proceed immediately!</i>
27		Spray with VLSI acetone and look to see no stray Pt is still attached.	<i>Proceed immediately!</i>
28		Spray entire wafer with VLSI IPA	<i>Proceed immediately!</i>
29		Single wafer spin dryer	
30	Inspecting Microscope	Ensure no platinum strips remain attached to electrodes.	<i>If not, return to step 6</i>

31	Measuring	Measure platinum on Chip #1	
32	Veeco Dektak 8	Measure platinum on Chip #6	

A.5 | Bonding of Glass Substrates

Step	Process	Parameters	Notes
1	Standard Cleaning	2min 100% HNO ₃ I	Removes any organic contaminants
2	Wetbench 16	Quick Dump Rinse	
3	Standard Cleaning Wetbench 17	15sec in 25% KOH @ 75°C dip	Etches slightly to achieve a more reproducible surface
4	Standard Cleaning	Immerse in DI H ₂ O container	Removes any particles attached to the surface
5	Wetbench 16	10min ultrasonic in the first rinse water	
6		Quick Dump Rinse	
7		1min 100% HNO ₃ I	Re-stabilises the glass surface.
8		1-2min 100% HNO ₃ II	
9		Quick Dump Rinse	
10		Leave in beaker filled with water until necessary for next step!	
11	Preparing Equipment Electronic Vision Group EV620 Mask Aligner	<ul style="list-style-type: none"> • Teflon Chuck • Pre-Bonding Holder • Manual Anodic Bonding • Crosshair • Flags before WEC 	Reduce the 'Wec' to almost zero, otherwise the wafers may stick already during the loading. If necessary, allow focusing again.
12	Drying Wetbench 16	Single wafer spin dryer Bottom wafer	
13	Loading Mask Aligner	Load front-side down and continue.	Alignment mark 1-1.
14	Drying Wetbench 16	Single wafer spin dryer Top wafer	

15	Pre-bonding Mask Aligner	Load top wafer front-side up and continue.	<i>Do not adjust Optical XY co-ordinates! Only Z.</i>
16		Carefully unload stack and inspect alignment under microscope.	<i>Return to step 7 if misaligned.</i>
17		Place on a black flat surface and rub with a synthetic tweezers to remove residual air. Air gaps appear as white or rainbow areas; pre-bond is transparent.	<i>Located in Jan's shelf of the Bios cabinet.</i>
18		Place in single wafer box.	
19	Bond Annealing TST Group	N ₂ gas oven in TST group <ul style="list-style-type: none">• 5hr ramping to 625°C• 1hr hold at 625°C• 10hr ramping to 25°C	<i>Nitrogen gas oven is used to limit oxidation of the Ta adhesion layer.</i>
20	Dicing Disco DAD dicing saw	<ul style="list-style-type: none">• TC300 blade• Height: 1.8mm• Transparent dicing foil (0.125mm)• Blade height: 0.05m• RPM: 25k	<ul style="list-style-type: none">• Speed: 4mm/s• Wafer size: 110mm• CH1 step: 15.3mm• CH2 step: 30.3mm

A.6 | Standard MESA+ Processes

Quick Dump Rinse

Two cycles of:

1. Fill bath – 5 seconds
2. Spray dump – 15 seconds
3. Spray fill – 90 seconds

End fill – 200 seconds

Continuous rinsing until DI water reads a resistivity of > 10MΩ

Single Wafer Spin Dryer

2500rpm – 60 seconds

Last 30 seconds, N₂ gas flowing

A.7 | Fabrication Challenges

Three iterations of the above process flow were required to successfully fabricate microchips which could be used for the experiments in this thesis.

Degradation of the electrodes was observed in the first fabricated microchips, as seen in Figure A.7-1a, where the top-right edge of one exposed electrode has been stripped away. While electrode degradation is primarily due to cross-talking detection and high voltage electronics when contact conductivity detection is used, the author noticed burn-like colour patterns on many microchips' thin film electrodes. These colour changes were attributed to the oxidation of the Ta adhesion layer (images in Figure A.7-1 were taken from the bottom-up). When the glass microchips are annealed in air, oxygen diffuses through the platinum layer, and oxidizes the Ta¹. It was not proven that the Ta oxidation

Figure A.7-1. Microscope images of fabricated microchips, taken from the bottom-up. In (a), colour differences can be seen along the electrodes (the Ta adhesion-layer is imaged), with a burn-like pattern at the glass/channel interface. In (b), discoloured patterns in the glass surrounding the etched channel indicates poor glass-bonding. (c) and (d) demonstrate imperfect channel etching due to surface roughness on the glass supplied by the manufacturer.

attributed to the oxidation of the Ta adhesion layer (images in Figure A.7-1 were taken from the bottom-up). When the glass microchips are annealed in air, oxygen diffuses through the platinum layer, and oxidizes the Ta¹. It was not proven that the Ta oxidation promoted slow electrode degradation when exposed to high DC voltages. However, the process flow was subsequently changed to anneal in a nitrogen gas oven to limit oxidation (step 19 of A.6), and a reduction in electrode damage was noted by the author thereafter.

The first iteration of microchips were fabricated using E. Vrouwe's masks² made for square wafers. In the time after his thesis project, the cleanroom facilities moved to using round wafers instead. While we attempted to fabricate the microchips on round wafers using the square masks anyway (which would result in the corner chips being lost), the microchip yield was dismally low due to the wafers cracking during processing and dicing. Thus, any poor bonding seen in the microchips was attributed to mask-wafer mismatch at this time.

After this initial run, the masks were redesigned for round wafers, while still keeping the individual microchip designs (e.g. channel lengths, widths, etc.). However, the second set of fabricated microchips also had poor bonding – much of which was calico-patterned and only visible using the transmitted light option on the Leica 6000M microscope, as pictured in Figure A.7-1b.

Thus, a third set of microchips were fabricated, and the KOH dip prior to bonding (step 3 of A.6) was increased from 10 to 15 seconds, to achieve a more reproducible surface for bonding. The final set of chips was better bonded; however, a new problem had emerged. The 1.1mm Borofloat stock supplied by the manufacturer was not sufficiently smooth, resulting in roughly etched channels (Figure A.7-1c) and/or deep scratches in an otherwise smooth channel (Figure A.7-1d). Time restraints in the project and the inference that these channel defects should not have significant weight on the final measurement, meant this was the final fabricated set of chips.

A.8 | References

1. Tiggelaar, R. M., Sanders, R. G. P., Groenland, A. W. & Gardeniers, J. G. E. Stability of thin platinum films implemented in high-temperature microdevices. *Sensors Actuators A Phys.* **152**, 39–47 (2009).
2. Vrouwe, E. X. Quantitative microchip capillary electrophoresis for inorganic ion analysis at the point of care. (2005).

APPENDIX B

experiment setup designs and pitfalls

B.1 | Basic Requirements

To successfully perform the experiments described in this thesis, the setup must consist of a few basic components:

High Voltage System

All four HV leads are utilised during both injection and separation steps, and so all HV ports on the system must be able to both *source* and *sink* current. Furthermore, the HV leads must all be tied to a common ground, which can then be tied to the building's earth/ground. This is important because the grounds on power outlets in the lab are usually common along the benches and are not tied to the building's earth, which can result in the HV system elevating the socket ground. By tying the HV system's common ground to earth, one can be sure that the voltage on the lead closest to the thin-film electrodes does, in fact, have zero potential with respect to the detector electronics. If it does not have zero potential, DC current will flow into the thin film electrodes, and will dissolve/degrade them.

Conductivity Measurement System

This is self-explanatory. Here, the detection electronics consist of a voltage divider and lock-in amplifier circuitry. For optimal sensitivity, care must be taken to match the measurement resistor value to the resistance of the microfluidic cell at the chosen AC detection frequency.

Separator Components

Pinched injection is performed in this thesis, and so the HV lead nearest the thin-film electrodes must be raised to at least 1kV during injection. If the detection electronics are connected to the chip at this time: at best, the thin-film electrodes will disappear; at worst, the conductivity meter will be damaged. Thus, either two switches or two relays are required to isolate the measurement system from the high voltage system. DC-blocking capacitors are not enough to isolate the two systems: the thin-film electrodes will be destroyed.

Since the thin-film electrodes are located a short distance from the waste reservoir, a potential of approximately 30V will exist between the thin-film electrodes in buffer and the detection electronics (ground) during separation. Thus, two HV DC-blocking capacitors (10nF was used here) must be placed between the chip holder and the detector electronics. On the AC voltage signal source, the capacitor should be placed as close to the chip as possible; i.e. directly between the ground-shielded cable and the chip holder. As the AC source is driven by the system, this will ensure the best quality signal is delivered to the chip. On the measurement signal (or meter input), however, the DC-blocking capacitor should be placed away from the chip holder; i.e. between the isolation switch/relay and the actively-guarded cable connecting the switch/relay to the holder. If the capacitor is located between the chip holder and the actively-guarded cable, then electrolysis will occur between the thin-film electrodes in the buffer solution. Why the capacitor's placement along the wire is so crucial was not investigated by the author; however, it is likely that the wire effectively increases the charging time constant of the capacitor, thereby preventing electrolysis.

Chip Holder

A three-piece Delrin® holder (see Figure B.1-1) consisting of a bottom support plate, a cover with reservoirs, and a top plate was designed for this thesis. The top plate interfaces the fluidic reservoirs to the HV system via four platinum wires and the conductivity electrodes to the detector electronics via two spring-loaded pins. The three piece design allowed for: the chip reservoirs to be extended from ~4 μ L to ~60 μ L; and, for the chip solutions to be easily changed without disconnecting each electrical lead from the chip holder each time (the top plate needs only to be lifted off).

Figure B.1-1. Photograph of the three-piece chip holder designed for this thesis project.

B.2 | Setup #1 – The Switch Box

The first successful setup designed during this thesis project (pictured in Figure B.2-1) consisted of:

high-voltage system – CU 411, IBIS Technologies, Hengelo, The Netherlands

detection electronics – Lock-In Conductivity Meter (in-house)

separator components – A little box with two push-button switches

Figure B.2-1. Diagram illustrating the switchbox experimental setup.

The author highly discourages the use of a manual switchbox if multiple measurements are to be performed. While the simplest solution in terms of hardware, this is incredibly inconvenient for the operator, and a momentary distraction will result in the destruction of the microfluidic chip.

B.3 | Setup #2 – Software-Controlled Relays

The second successful setup designed during this thesis project, and used for the experiments measured in Chapters 3 and 4, consisted of:

high-voltage system – CU 411, IBIS Technologies, Hengelo, The Netherlands

detection electronics – Lock-In Conductivity Meter (in-house)

separator components – Four switches and an Arduino Uno microcontroller board

An Arduino Uno microcontroller board was used to facilitate switching of the relays with its 5V DC pins. PhotoMOS relays were implemented on the first design iteration; however, while rated for high voltages, they were not rated for the high AC frequencies used in this thesis (and were basically like wire). Thus, high-vacuum reed switches (cynergy3 D-Series 15kV, 50W, Tungsten contacts, 12V coil) were subsequently used. To operate them with the Arduino Uno's 5V pins, MOSFETs (Harris IRF610) were used to facilitate switching (Figure B.3-1) and the 12V supply necessary to actuate the switches was powered externally.

Figure B.3-1. Switch circuitry used to isolate the detector system from the high voltage supply. +5V is sourced by the Arduino Uno and the +12V is sourced by an external supply. The diode is added to protect the switch when the Arduino Uno is not supplying voltage.

It should be noted that the 12V supply must have its ground tied to the building's earth. Without this, the author found cross-talking electronics in the power supply would cause the switches to rapidly oscillate between being actuated and not.

The author also found that the switches must be operated in air. When a simple breadboard PCB was used, electrolysis was observed between the thin-film electrodes (possibly due to some kind of leakage on the board). Thus, a metal box was fitted to hold the switches, MOSFETs, and wires (Figure B.3-2). Two additional switches were also added to the box, so a precision resistor could be measured during the injection step. It was the author's intention to see if these measurements could be used to correct for changes in the system environment; unfortunately, time constraints prevented this.

Figure B.3-2. Schematic of the metal box (pseudo Faraday Cage) designed to hold the switches in air. 12V and ground pins are connected to an external supply not drawn here. The 'R' designates the precision resistor.

B.4 | Setup #3 – New HV System

The third setup designed during this thesis project, and used for the experiments measured in Chapters 5, consisted of:

high-voltage system – HVS448 3000-LC, LabSmith Inc., Livermore CA, USA

detection electronics – Lock-In Conductivity Meter (in-house)

separator components – Four switches and an Arduino Uno microcontroller board

The high voltage system was changed for a number of reasons. One, the LabSmith system is capable of higher voltages (max. 3kV, compared to the IBIS' 1kV); higher voltages can improve the electrophoretic measurement's resolution. Two, it is capable of recording the current on all channels – something the author attempted to implement in the IBIS, but never worked properly. Lastly, and most importantly, the author observed a strange "accordion"-like noise during some measurement that appeared and disappeared at whim. The noise coupled to the signal only appeared during separation, and would start at a frequency of 40~60Hz, stretch to a wobbling frequency of ~10Hz, before increasing again, and then disappearing. Restarting the equipment didn't seem to have an effect. During Chapter 3, the noise would appear rarely – once or twice in 8-9 hours of experiments. However, while trying to perform experiments for Chapter 5, the noise began appearing more frequently – once every 1-2 hours. Speculating that perhaps some components in the IBIS were failing due to the system's age and because the noise only appeared during separation, the high voltage system was replaced.

Unfortunately, the author found out after implementing the new setup, that this didn't eliminate the accordion-like noise at all – it persisted over the course of collecting experiments for Chapter 5, before very randomly and suddenly changing to appearing only while measuring the precision resistor and not the microfluidic cell. Upon inspection with an oscilloscope, all systems appeared to be working properly, and so this was one mystery of this thesis which remained unsolved.

Two more issues had to be solved when changing the high voltage supply. Firstly, the software used to simultaneously operate the isolation switches and applied HV needed to be changed. LabSmith only offers LabView drivers and a MS Visual Studio software developer's kit – the latter of which was poorly documented. To use the system with the author's Python code written for Setup #2, the MS Visual Studio files needed to be wrapped into a Python library.

Secondly, and more relevant to this appendix, a low-frequency signal was coupled into the measurement signal after the HV supply was changed (Figure B.4-1a). Unlike the "accordion" noise, this signal was persistent, and the author determined it was coming from the LabSmith system. Connecting a DC-blocking capacitor (10nF) from the HV lead at the waste reservoir to measurement earth resulted in electrolysis between the thin-film electrodes. The author speculated this was due to the discharging of the capacitor between injection (1kV) and separation (GND). As described in Section B.1, it was previously found that adding a bit of shielded wire could alter the time-constant of the capacitor charging – and the author found that, indeed, adding a bit of shielded cable

between the chip holder and the capacitor corrected the electrolysis in this instance as well. Here, the optimal wire length was found to be ~2cm (Figure B.4-1b). Lengths shorter than this resulted in the DC-blocking capacitor sinking too much of the AC measurement signal, thereby reducing sensitivity. Conversely, longer wire lengths would not effectively sink the HV-source noise, disrupting the measurement.

Figure B.4-1. Electropherograms demonstrating (a) the low-frequency signal from the LabSmith system, and (b) the resultant electropherogram when a DC-blocking capacitor and 2cm of shielded wire was added to the HV lead nearest the thin-film electrodes.

APPENDIX C

software and signal processing code

C.1 | Arduino Uno Firmware

```
/*
Relay Code

Monitors the serial port and changes four relays based on user input.

Microchip Relays
Relay 1: Pin 2 --> R --> GND
Relay 2: Pin 3 --> R --> GND

Reference Resistor Relays
Relay 1: Pin 5 --> R --> GND
Relay 2: Pin 6 --> R --> GND

created 11 Apr 2013
by Allison C.E. Bidulock
BIOS Lab on a Chip Group
University of Twente, Netherlands
*/

char inChar = ' ';

void setup()
{
  DDRD = DDRD | B01101100; // sets pins as outputs, without modifying other pins
  PORTD = B00000000;       // sets all outputs to LOW
  Serial.begin(9600);      // open serial port
  Serial.println("Program Initiated");
}
```

```

void loop()
{
    while (Serial.available()>0) {
        inChar = Serial.read();
    }

    if (inChar=='0') {
        PORTD = B00000000;    // sets all outputs to LOW
        Serial.println("Relays Switched (F)");
        inChar = ' ';
    }
    else if (inChar=='1') {
        PORTD = B00001100;    // sets relays to measure microchip
        Serial.println("Relays Switched (M)");
        inChar = ' ';
    }
    else if (inChar=='2') {
        PORTD = B01100000;    // sets relays to measure reference
        Serial.println("Relays Switched (R)");
        inChar = ' ';
    }
}

```

C.2 | Python Code: IBIS

[Chapters 3-4]

```

import serial
import time

runcount = 5                # Number of Inj-Sep to do (integer)
injtime = [60,45,45,45,45]  # Length of Inj (s)
septime = [45,45,45,45,45]  # Length of Sep (s)

# Reverse Engineered commands for different voltage settings to the IBIS
# Vinj: 1(100V) 2(800V) 3(0V) 4(1000V)
Vinj = '\x50\x05\x00\x0B\xff\x7F\x66\x66\x00\x00\xff\x7F\x0F\x00\x37'
# Vsep: 1(600V) 2(1000V) 3(600V) 4(0V)
Vsep = '\x50\x05\x00\x0B\xCC\x4C\xff\x7F\xCC\x4C\x00\x00\x0F\x00\x1D'
Von = '\xFD\x02\x0C\x00\x0B\x01\x52\x00\x64\x00\xCD\x01'
Voff = '\x50\x05\x00\x0B\x00\x00\x00\x00\x00\x00\x00\x00\x00\x00\x60'

# Arduino firmware commands for Relay settings
Rinj = '2'    # Precision R
Rsep = '1'    # Microchip
Roff = '0'    # All open

# Try if the system is able to open a COM port to the Arduino
try:
    arduino = serial.Serial('COM5',9600) # Baudrate=9600
    print arduino.readline()    # Should receive "Program Initiated"
except:
    print "Failed to connect to Arduino."

```

```

# Try if the system is able to open a COM port to the IBIS
try:
    # Baudrate=38400, parity=even
    ibis = serial.Serial('COM1',baudrate=38400,timeout=10,
        parity=serial.PARITY_EVEN)
    # Initialize command necessary to communicate with IBIS
    ibis.write(Von)
    # Software receives a 14 character response
    # empty the buffer by reading them
    print ibis.read(14)
except:
    print "Failed to connect to IBIS."

# If both COM ports are connected, then proceed with the experiment
if ((arduino.isOpen() is True) and (ibis.isOpen() is True)):

    # Repeat Inj-Sep for the number of runcounts
    for i in range(0,runcount,1):

        # Record the current time and turn on the HV
        now = time.time()
        ibis.write(Vinj)

        # Switch relays to Inj and pause for 250ms until they switch
        time.sleep(1.0)
        arduino.write(Rinj)

        # Send HV commands to the IBIS every sec until the time is up
        j = 0
        while (time.time()-now < injtime[i]-0.5):
            time.sleep(0.1)
            j = j+1
            if (j == 9):
                ibis.write(Vinj)
                print "Injecting... %.1f" % (time.time()-now)
                j = 0

        # Open Relays and sleep for remainder of Inj Time
        arduino.write(Roff)
        time.sleep(0.5)

        # Record the current time and change the HV to Sep
        now = time.time()
        ibis.write(Vsep)

        # Pause for 500ms to ensure HV=GND before switching relays
        time.sleep(1.0)
        arduino.write(Rsep)

        # Send HV commands to the IBIS every sec until the time is up
        j = 0
        while (time.time()-now < septime[i]-0.5):
            time.sleep(0.1)
            j = j+1

```

```

        if (j == 9):
            ibis.write(Vsep)
            print "Separating... %.1f" % (time.time()-now)
            j = 0

    # Open Relays and sleep for remainder of Sep Time
    arduino.write(Roff)
    time.sleep(0.5)

    # Once all runs are complete, open relays and turn off HV
    print "Closing..."
    ibis.write(Voff)

    # Close both COM Ports
    ibis.close()
    arduino.close()
    print "Ports closed."

```

C.3 | Python Code: LabSmith

[Chapter 5]

```

import serial
import time
import HVS448

runcount = 5                # Number of Inj-Sep to do (integer)
injtime = [45,45,45,45,45]  # Length of Inj (s)
septime = [35,35,35,35,35]  # Length of Sep (s)

# Voltage sequence must first be programmed via the LabSmith software.
# Once programmed, the EEPROM remembers the sequence even when powered off.
# StepA(0): A(1000V) B(800V) C(0V) D(1000V) - Injection
# StepB(1): A(975V) B(1500V) C(950V) D(0V) - Separation
hvs = HVS448.HVS()

# Arduino firmware commands for Relay settings
Rinj = '2'    # Precision R
Rsep = '1'    # Microchip
Roff = '0'    # All open

# Try if the system is able to open a COM port to the Arduino
try:
    arduino = serial.Serial('COM5',9600) # Baudrate=9600
    print arduino.readline() # Should receive "Program Initiated"
except:
    print "Failed to connect to Arduino."

# Try if the system is able to open a COM port to the LabSmith
hvsOpen = hvs.initConnection(1)
if (hvsOpen != 0): print "Failed to connect to LabSmith."

```

```

# If both COM ports are connected, then proceed with the experiment
if ((arduino.isOpen() is True) and (hvsOpen == 0)):

    # Prepare the LabSmith for experiments
    time.sleep(0.2)
    hvs.enableHV()
    time.sleep(0.2)
    chanA = hvs.getChannel("A")
    chanB = hvs.getChannel("B")
    chanC = hvs.getChannel("C")
    chanD = hvs.getChannel("D")

    # Get the current time, and name the current data file with it
    now = time.strftime('%y.%m.%d_%H%M%S')
    datafile = open(str(now)+'_LabSmith.txt','a')

    # Repeat Inj-Sep for the number of runcounts
    for i in range(0,runcount,1):

        # Record the current time and turn on the HV
        now = time.time()
        hvs.runStep(0)

        # Record current data @ 10Hz until the time is up
        j = 0
        relayOn = 0
        timeskip = 0
        while (timeskip < injtime[i]):

            # Get time elapsed and current data, then write to txt file
            timeskip = time.time()-now
            uAdata = "%.3f" % (timeskip) \
                    + '\t' + str(chanA.getMeasurement()['current']) \
                    + '\t' + str(chanB.getMeasurement()['current']) \
                    + '\t' + str(chanC.getMeasurement()['current']) \
                    + '\t' + str(chanD.getMeasurement()['current']) + '\n'
            datafile.write(uAdata)

            # Print to the Python Window only every ~1 second.
            if (j == 9):
                print "Inj " + uAdata
                j = 0
            j = j+1

        # Let HV equilibrate in the chip before switching relays Inj
        if (timeskip > 0.5 and timeskip < injtime[i]-1.0
            and relayOn == 0):
            arduino.write(Rinj)
            relayOn = 1

        # Open Relays and write current for remainder of Inj Time
        if (timeskip > injtime[i]-1.0 and relayOn == 1):
            arduino.write(Roff)

```

```

        relayOn = 0

        # Sleep to bring intervals to ~100ms
        time.sleep(0.085)

    # Record the current time and change the HV to Sep
    now = time.time()
    hvs.runStep(1)

    # Record current data @ 10Hz until the time is up
    j = 0
    relayOn = 0
    timeskip = 0
    while (timeskip < septime[i]):

        # Get time elapsed and current data, then write to txt file
        timeskip = time.time()-now
        uAdata = "%.3f" % (timeskip) \
                + '\t' + str(chanA.getMeasurement()['current']) \
                + '\t' + str(chanB.getMeasurement()['current']) \
                + '\t' + str(chanC.getMeasurement()['current']) \
                + '\t' + str(chanD.getMeasurement()['current']) + '\n'
        datafile.write(uAdata)

        # Print to the Python Window only every ~1 second.
        if (j == 9):
            print "Sep " + uAdata
            j = 0
        j = j+1

        # Let HV equilibise in the chip before switching relays Sep
        if (timeskip > 0.5 and timeskip < septime[i]-1.0
            and relayOn == 0):
            arduino.write(Rsep)
            relayOn = 1

        # Open Relays and write current for remainder of Sep Time
        if (timeskip > septime[i]-1.0 and relayOn == 1):
            arduino.write(Roff)
            relayOn = 0

        # Sleep to bring intervals to ~100ms
        time.sleep(0.085)

# Once all runs are complete, open relays and turn off HV
print "Closing..."
hvs.stop()
hvs.disableHV()

# Close both COM Ports
hvs.closeConnection()
arduino.close()
datafile.close()
print "Ports closed."

```


C.4 | MATLAB Code: LockIn2Mat

[Chapters 3-5]

```
% LockIn CSV Files to MatLab MAT Files
%-----
% Converts LockIn experiment files (one file typically contains multiple runs)
% into matlab data files; one file for each run in one experiment.
%
% created 14 Jan 2014
% by Allison C.E. Bidulock
% BIOS Lab on a Chip Group
% University of Twente, Netherlands
%-----

clear all
offv = 0.02; %Vrms

% request directory to get all files from
folderName =
uigetdir('\ad.utwente.nl\home\BidulockACE\Documents\Experiments\');
fileList = getAllFiles(folderName);

for i=1:length(fileList);

    [pathName, fileName, ext] = fileparts(fileList{i,1});

    % if the file is a LockIn file, then get all data
    if isequal(ext, '.csv')
        lockinData = importdata(fileList{i,1});

        % split into magnitude and phase data
        dataMg = lockinData(250:end,1);
        dataPh = lockinData(250:end,2);

        % separate data into separate files for each run in the experiment
        m=1; k=1;
        while ((length(dataMg)-m) > 10)

            % find falling edge
            while ((dataMg(m) < offv) && ((length(dataMg)-m) > 10))
                m = m+1;
            end
            % find rising edge
            j = m;
            while ((dataMg(j) > offv) && ((length(dataMg)-j) > 10))
                j = j+1;
            end

            if ((length(dataMg)-j) > 10)
                % snip off some excess
                mg = dataMg(m+10:j-10);
                ph = dataPh(m+10:j-10);

                % determine if it's a reference or experiment run and save
```

```

        if isequal(mod(k,2),1)
            matFile = [fileName, '_ref', int2str((k+1)/2), '.mat'];
        end
        if isequal(mod(k,2),0)
            matFile = [fileName, '_run', int2str((k)/2), '.mat']
        end
        save([pathName, '\', matFile], 'mg', 'ph');
        k = k+1;
        m = j;
    end
end
end
i = i+1;
end

```

C.5 | MATLAB Code: Mat2Png (1)

[Chapter 3]

```

% MatLab MAT Files to Processed Files
%-----
% Converts matlab data files into images and collects all processed data into
% one matlab file. Experiment folder hierarchy creates image names.
%
% created 14 Jan 2014
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%-----

function [pkTotal] = Mat2Png1

    clear all;

    % request directory to get all files from
    folderName = uigetdir(removed);
    fileList = getAllFiles(folderName);
    pkTotal = [];

    for i=1:length(fileList);

        [pathName, fileName, ext] = fileparts(fileList{i,1});
        a = fileList{i,1}

        % if the file is reference mat file, then go to the reference function
        if isequal(fileName(end-3:end-1), 'ref') && isequal(ext, '.mat')
            processRef(pathName, fileName)
        end
        % if the file is an electropherogram mat file, then go to run function
        if isequal(fileName(end-3:end-1), 'run') && isequal(ext, '.mat')
            peaks = processRun(pathName, fileName);
            pkTotal = [pkTotal; peaks];
            save([folderName, '\data_medp1.mat'], 'pkTotal');
        end
    end
end

```

```

        end
        i = i+1;
    end

    header = 'Chip-Conc-Load-Run-Peak#-Height(Arb)-Area(PHMW)-Area(trapz)-
Time(ind)-Time(PHMW)-Time(trapz)';
    header = strread(header,'%s','delimiter','-');
    pkTotal = [header';pkTotal];
    save([folderName,'\data_medp1.mat'],'pkTotal');
end

function processRef(pathName,fileName)
    a = 1;
end

function peaks = processRun(pathName,fileName)
    % experiment details
    expSet = 'IS vs CC (IS)';
    bge = '100mM MES-His .01% HPMC .25mM KCl';
    istd = '7.5mM Cs,Li';
    sbge = '100mM MES-His .01% HPMC';
    dataRate = 238.42; %Hz    %datarate = 476.84; %Hz
    csAvg = 6.26036964;
    liAvg = 10.43396807-csAvg;

    % processing details
    fNorm = 12/(dataRate/2);
    [b,a] = butter(5, fNorm, 'low');

    % generate run information based on folder path
    folders = strread(pathName,'%s','delimiter','\\');
    conc = folders{end,1};
    chip = folders{end-1,1};
    sample = ['S: ',conc,' Na ',istd,' ',sbge];
    plotTitle = {[expSet,' | ',sample],['Chip ',chip,' | BGE: ',bge]};

    % load experiment parameters from the mat file
    load([pathName,'\',fileName,'.mat']);
    timeSp = 1/dataRate;
    time = ([0:1:(length(mg)-1)])*timeSp;

    % filter the magnitude data
    mgf = filtfilt(b, a, mg);

    % run a median subtraction on the filtered data
    k=175;
    m = mgf;
    for i=k+1:1:(length(mg)-k)
        m(i,1)=median(mgf(i-k:i+k,1));
    end

    % build plot windows with main data
    % save the full run and zoom in on the peaks
    f1 = figure(1);

```

```

set(gcf,'Visible','off');
plotName = [pathName,'\ ',fileName];
full = 1;
plotRun(f1,plotName,plotTitle,time,[mgf,m],full);

% set up figure 2 to have the processed lines
% process peak data
f2 = figure(2);
set(gcf,'Visible','off');
plotName = [pathName,'\ ',fileName,'-pro'];
full = 0;
data = mgf-m;
plotRun(f2,plotName,plotTitle,time,data,full);
peaks = findPeaks(chip,conc,fileName,time,data,liAvg);
saveas(f2,[plotName,'.png'],'png')

```

end

```

function [f1,f2] = plotRun(f,plotName,plotTitle,x,y,full)
clf;
set(gcf, 'DefaultAxesColorOrder', [102/256 194/256 165/256;...
                                   252/256 141/256 98/256;...
                                   141/256 160/256 203/256; 231/256 138/256 195/256; ...
                                   166/256 216/256 84/256; 255/256 217/256 47/256]);

plot(x,y);
hold all
xlabel('Time [s]', 'FontSize', 8, 'Color', [75/256 75/256 75/256]);
ylabel('Voltage [V] [V_r_m_s]', ...
       'FontSize', 8, ...
       'Color', [75/256 75/256 75/256]);
title(plotTitle, 'FontSize', 10);
set(gca, ...
      'FontSize', 8, ...
      'XColor', [75/256 75/256 75/256], ...
      'YColor', [75/256 75/256 75/256], ...
      'Box'      , 'off'      , ...
      'TickDir'  , 'out'     , ...
      'TickLength', [.01 .01] , ...
      'XMinorTick', 'on'     , ...
      'YMinorTick', 'on'     , ...
      'YGrid'    , 'on'     , ...
      'LineWidth' , 1        );
set(f,'PaperUnits','inches','PaperPosition',[0 0 6 4.5])
if isequal(full,1)
    saveas(f,[plotName,'-zoom.png'],'png')
end
xlim([5 15]);
saveas(f,[plotName,'.png'],'png')

```

end

```

function [peaks] = findPeaks(chip,conc,fileName,time,data,liAvg)
peaks = [];
runNum = fileName(end);
test = strread(fileName,'%s','delimiter','_');

```

```

expNum = [test{1,1}, '_', test{2,1}];

% Find peaks and build peak matrix
% 1Chip 2Conc 3Load 4Run 5Peak#
% 6Height(Arb) 7Area(FWHM) 8Area(trapz)
% 9Time(ind) 10Time(FWHM) 11Time(trapz)
[maxtab, mintab] = peakdet(data, 0.0005);
peak_indexs = maxtab(:, 1);

% If the first peak is the start of the electropherogram, toss it
if isequal(peak_indexs(1), 1)
    peak_indexs = peak_indexs(2:end);
end

% If the first peak is too early for Cs, toss it
if time(peak_indexs(1)) < 5
    peak_indexs = peak_indexs(2:end);
end

for i = 1:length(peak_indexs)

% Save experiment/run information and height/time
    peaks{i, 1} = chip; peaks{i, 2} = conc;
    peaks{i, 3} = expNum; peaks{i, 4} = runNum;
    peaks{i, 5} = i;
    peaks{i, 9} = time(peak_indexs(i));
    peaks{i, 6} = data(peak_indexs(i));

% Determine half-width indices, plot and save
    j = peak_indexs(i);
    while data(j) > (peaks{i, 6}/2)
        j = j - 1;
    end
    k = peak_indexs(i);
    while data(k) > (peaks{i, 6}/2)
        k = k + 1;
    end
    peaks{i, 7} = 1.0646 * peaks{i, 6} * (time(k) - time(j));
    peaks{i, 10} = time(j);
    plot([time(j), time(k)], [data(j), data(k)]);

% Determine full-width indices, plot and save
    while data(j) > 0
        j = j - 1;
    end
    while data(k) > 0
        k = k + 1;
    end
    peaks{i, 8} = trapz(time(j:k), data(j:k));
    plot([time(j), time(k)], [data(j), data(k)]);
    peaks{i, 11} = time(j);
end
end

```

C.6 | MATLAB Code: Mat2Png (2)

[Chapter 4] - only relevant sections which were modified from Mat2Png(1)

```
function peaks = processRun(pathName,fileName)
    [...]

    % filter the magnitude data and remove electropherogram after 'Li' peak
    mgf = filtfilt(b, a, mg);

    if (length(mgf) > 4508)
        mgfsub = mgf(1240:4508);
        timesub = time(1240:4508);
    else
        mgfsub = mgf(1240:end);
        timesub = time(1240:end);
    end

    m = backcor(timesub,mgfsub,5,0.01,'sh');

    [...]
end

function [peaks] = findPeaks(chip,conc,fileName,time,data)

    peaks = [];
    runNum = fileName(end);
    test = strread(fileName,'%s','delimiter','_');
    expNum = [test{1,1}, '_',test{2,1}];

    % Find peaks and build peak matrix
    % 1Chip 2Conc 3Load 4Run 5Peak#
    % 6Height(Arb) 7Area(FWHM) 8Area(FWHM.1) 9Area(medm) 10Area(medm.1)
    % 11Time(ind) 12Time(FWHM) 13Time(trapz)
    [maxtab,mintab]=peakdet(data,0.002);
    peak_indexs=maxtab(:,1);
    [maxtab,mintab]=peakdet(data,0.001);
    peak_indexs2=mintab(:,1);

    % If the first peak is the start of the electropherogram, toss it
    if isequal(peak_indexs(1),1)
        peak_indexs = peak_indexs(2:end);
    end

    % If the first peak is too early for Na, toss it
    if time(peak_indexs(1)) < 7
        peak_indexs = peak_indexs(2:end);
    end

    for i=1:length(peak_indexs)

    % Save experiment/run information and height/time
        peaks{i,1}=chip; peaks{i,2}=conc;
        peaks{i,3}=expNum; peaks{i,4}=runNum;
```

```

        peaks{i,5}=i;
        peaks{i,11}=time(peak_indexs(i));
        peaks{i,6}=data(peak_indexs(i));
        halfh = peaks{i,6}/2;

% Determine half-width indices, plot and save
        j=peak_indexs(i);
        while data(j)>halfh
            j = j-1;
        end
        tleft=((halfh-data(j))*(time(j+1)-time(j))/(data(j+1)-
data(j)))+time(j);
        k=peak_indexs(i);
        while data(k)>halfh
            k = k+1;
        end
        tright=((halfh-data(k))*(time(k-1)-time(k))/(data(k-1)-
data(k)))+time(k);
        peaks{i,7}=1.0646*peaks{i,6}*(tright-tleft);
        peaks{i,9}=trapz(time(j+1:k-1),data(j+1:k-1))+(0.5*(data(j+1)-
halfh)*(time(j+1)-tleft))+(0.5*(data(k-1)-halfh)*(tright-time(k-
1)))+(halfh*(tright-tleft));
        peaks{i,12}=tleft;
        plot([tleft, tright], [halfh, halfh]);

% Determine full-width indices, plot and save
        halfh=peaks{i,6}/10;
        while data(j)>halfh
            j = j-1;
        end
        tleft=((halfh-data(j))*(time(j+1)-time(j))/(data(j+1)-
data(j)))+time(j);
        k=peak_indexs(i);
        while data(k)>halfh
            k = k+1;
        end
        tright=((halfh-data(k))*(time(k-1)-time(k))/(data(k-1)-
data(k)))+time(k);
        peaks{i,8}=peaks{i,6}*(tright-tleft);
        peaks{i,10}=trapz(time(j+1:k-1),data(j+1:k-1))+(0.5*(data(j+1)-
halfh)*(time(j+1)-tleft))+(0.5*(data(k-1)-halfh)*(tright-time(k-
1)))+(halfh*(tright-tleft));
        peaks{i,13}=tleft;

    end

    for f=i+1:1:length(peak_indexs2)+i

% Save experiment/run information and height/time
        peaks{f,1}=chip; peaks{f,2}=conc;
        peaks{f,3}=expNum; peaks{f,4}=runNum;
        peaks{f,5}=f;
        peaks{f,11}=time(peak_indexs2(f-i));
        peaks{f,6}=data(peak_indexs2(f-i));
        halfh = peaks{f,6}/2;

```

```

% Determine half-width indices, plot and save
j=peak_indexs2(f-i);
while data(j)<halfh
    j = j-1;
end
tleft = ((halfh-data(j))*(time(j+1)-time(j)))/(data(j+1)-
data(j))+time(j);
k=peak_indexs2(f-i);
while data(k)<halfh
    k = k+1;
end
tright = ((halfh-data(k))*(time(k-1)-time(k)))/(data(k-1)-
data(k))+time(k);
peaks{f,7}=1.0646*peaks{f,6}*(tright-tleft);
peaks{f,9}=trapz(time(j+1:k-1),data(j+1:k-1))+(0.5*(data(j+1)-
halfh)*(time(j+1)-tleft))+(0.5*(data(k-1)-halfh)*(tright-time(k-
1)))+(halfh*(tright-tleft));
peaks{f,12}=tleft;
plot([tleft, tright], [halfh, halfh]);

% Determine full-width indices, plot and save
halfh=peaks{f,6}/10;
while data(j)>halfh
    j = j-1;
end
tleft=((halfh-data(j))*(time(j+1)-time(j)))/(data(j+1)-
data(j))+time(j);
while data(k)>halfh
    k = k+1;
end
tright=((halfh-data(k))*(time(k-1)-time(k)))/(data(k-1)-
data(k))+time(k);
peaks{f,8}=peaks{f,6}*(tright-tleft);
peaks{f,10}=trapz(time(j+1:k-1),data(j+1:k-1))+(0.5*(data(j+1)-
halfh)*(time(j+1)-tleft))+(0.5*(data(k-1)-halfh)*(tright-time(k-
1)))+(halfh*(tright-tleft));
peaks{f,13}=tleft;

end
end

```

C.7 | MATLAB Code: Mat2Png (3)

[Chapter 5] - only relevant sections which were modified from Mat2Png(1)(2)

```

function peaks = processRun(pathName,fileName)
[...]
```

% filter the magnitude data and remove electropherogram after 'Li' peak

```

mgf = filtfilt(b, a, mg);

mgfsub1 = mgf(250:2550);

```



```

timesub1 = time(250:2550);
m1 = backcor(timesub1,mgfsub1,3,0.001,'sh');

[...]
end

function [peaks] = findPeaks(chip,conc,fileName,time,data)
[...]

[maxtab,mintab]=peakdet(data,0.00025);
peak_indexs=maxtab(:,1);
peak_indexs2=mintab(:,1);

% If the first peak is the start of the electropherogram, toss it
if isequal(peak_indexs(1),1)
    peak_indexs = peak_indexs(2:end);
end

% If the first peak is too early for K, toss it
if time(peak_indexs(1)) < 4
    peak_indexs = peak_indexs(2:end);
end

% If the second peak is too late for Na, toss it
if length(peak_indexs) > 2
    if time(peak_indexs(3)) > 8
        peak_indexs = peak_indexs(1:2);
    end
end

% If the first peak is too early for Cs, toss it
if time(peak_indexs2(1)) < 3
    peak_indexs2 = peak_indexs2(2:end);
end

% If the second peak is too late for Cs, toss it
if length(peak_indexs2) > 1
    if time(peak_indexs2(2)) > 5
        peak_indexs2 = peak_indexs2(1);
    end
end

[...]
end

```

OUTPUT

Peer-Reviewed Journal Publications

Bidulock, A.C.E., van den Berg, A., Eijkel, J.C.T., "Improving chip-to-chip precision in disposable microchip capillary electrophoresis devices with internal standards", *Electrophoresis* 2015, 36, 875-883.

Bidulock, A.C.E., van den Berg, A., Eijkel, J.C.T., "Improved Quantification For Point-Of-Care Capillary Electrophoresis By Incorporating An Internal Standard In The Background Electrolyte – I. Simple Sample Matrix", in preparation.

Bidulock, A.C.E., van den Berg, A., Eijkel, J.C.T., "Improved Quantification For Point-Of-Care Capillary Electrophoresis By Incorporating An Internal Standard In The Background Electrolyte – II. Sample Matrix with Controlled Composition", in preparation.

Patents

Bidulock, A.C.E., Eijkel, J.C.T., "Background-electrolyte internal standard addition for electrophoresis", U.S. Patent Application No. US62/14637 (2015).

International Conferences

Bidulock, A.C.E., van den Berg, A., Eijkel, J.C.T., "Improved precision using internal standards in disposable point-of-care capillary electrophoresis devices", In: 18th International Conference on Miniaturized Systems for Chemistry and Life Sciences, MicroTAS 2014, 26-30 Oct 2014, San Antonio, TX, USA.

Bidulock, A.C.E., van den Berg, A., Eijkel, J.C.T., "improved quantification for point-of-care capillary electrophoresis by incorporating an internal standard in the background electrolyte", In: 19th International Conference on Miniaturized Systems for Chemistry and Life Sciences, MicroTAS 2015, 25-29 Oct 2015, Gyeongju, South Korea.

ABSTRACT

To realise portable microchip capillary electrophoresis systems for routine use in point-of-care (POC) settings, measurements must be robust across many single-use chips. While it is well-known that internal standards (ISTDs) improve run-to-run reproducibility in conventional analytical chemistry methods, this thesis explores ISTDs in two novel ways.

First, two ISTDs are used in a systematic investigation to determine the significance of chip-to-chip imprecision. Six basic, in-house fabricated microchips were used to determine the reproducibility of NaCl quantification with two ISTDs: CsCl and LiCl. Chip-to-chip imprecision was significantly larger than intrachip. We attributed this to a significant variation in microchannel surface properties, while microchip dimension and detector variations were small in comparison. Cs and Li ISTDs both corrected the chip-to-chip precision; however, Cs was clearly superior, suggesting that the ISTD must be carefully chosen as in conventional methods.

In a second investigation, the same experiments were performed again – except with the ISTDs added to the background electrolyte (BGE) instead of the sample, resulting in the formation of two system peaks. While this phenomena is well-known, intentionally adding a system peak for use as a quantitative reference standard has yet to be reported – perhaps due to the availability of traditional ISTDs in conventional laboratories. When CsCl and LiCl were added to the BGE, the chip-to-chip precision of Na quantification improved without using the reference peak, and was further reduced when Cs or Li was used as a correction factor. Furthermore, both Cs and Li performed comparably well, suggesting that the method was more robust and less selective than a traditional ISTD under these simple experimental conditions.

Finally, the method was further investigated using a more-complex and less-varying sample matrix, with cation concentrations similar to human blood. The BGE-added "Cs" system peak improved the measurement of clinically relevant K concentrations and resulted in linear calibration curves. While further investigation is necessary, the novel method proposed in this thesis gave promising results and is attractive for use in POC settings due to its simplicity: no additional sample manipulation, microchip changes, or system expansions are required for implementation.

ACKNOWLEDGEMENTS

this thesis was made possible thanks to many wonderful people

Welcome, welcome, to the end of this dissertation – or for many of you, the beginning! I assure you, since you are holding this book in your hands, the mystery of whether the author completes her thesis or not has already been spoiled (there may have been a few breath-holding moments, but you can move back from the edge of your seat now...). And if you're looking for a "vine" version of the entire text, I'll refer you one page earlier, where I've somehow managed to condense 100+ pages into 350 words or less!

But this is no mystery novel, nor is it a social media exchange (or I definitely would have included kitty gifs), and though I can poke fun at you, I can't fault you if you're reading this section first: here lies the true heart behind what has made this thesis possible. (The rest is *only* science...)

To my promoter, Albert: I am deeply grateful for the opportunity to work at BIOS. You lead a group that is as personally interconnected as it is large, and is a very welcome place for many PhD students over the course of their four years. Our meetings guided me to hold firmly onto the broader picture of the project, while also considering small details of my work, and helped me to maintain a better balance in my progress. The first conference we attended, you made a note of coming to visit my poster at the start of the session, and really helped settle my nerves by going through it with me step-by-step. Knowing how busy you were, this moment was very special to me, and is one I won't forget.

To my other promoter and daily supervisor, Jan: I'm not sure if I'm able to express my gratitude in a few simple sentences, but I'll try. From the very first Skype call we had – even before my interview or the start of my project – you have been encouraging me with your kindness and consideration. You pushed gently at my back when I needed to jump

start (or put the "completed" stamp...) on something, helped me unsnarl all of my frustrations towards my temperamental setup, and always saw the positives in my work when I couldn't see them myself. At first, the near-complete freedom to control the direction of my project gave me many shaky moments, but I'm very grateful you allowed me the opportunity to drive myself towards my own destination, and acted as a guide instead of a map. I am confident, and have been since the day I first met you in person, that I could not have asked for a better supervisor.

My setup for all of the experiments in this thesis is primarily working due to the patient and persistent help from Ad and Mathieu. Even though my project went into more of an analytical chemistry direction in the end, I developed my electrical engineering skills thanks to these two very knowledgeable people. Ad, thank you for all your help in ramping up with the equipment and going through many design revisions (a few conductivity meters, a few PCBs, many fried chips later...) with me, even when your time at the university was so limited. I enjoyed hearing your stories of finicky and random technical problems (but I know barely 10 words in French, honest).

Mathieu, thank you for being my mentor, my colleague, and my friend. Even though I had an MSc in EE, you patiently sat with me, teaching me how to solder without making fun of me for not knowing how. On so many occasions, you listened patiently to my elaborate (and likely, sometimes near-hysterical) stories of ghosts and phantom noise coming out of my system, and always had a new solution to test or something to investigate to try and solve it. You encouraged me like a supervisor, and listened to my worries as a friend when I needed sound advice. Truly, thank you for everything.

Johan, you helped me not only in getting a working process flow, but also painstakingly in fabricating chips. When we used Elwin's square masks, I remember you carefully trying to process fragments of the wafers which broke on etching, when I probably would have been knocking my head against the wetbench glass in frustration. At the end, when I was so desperate to get well-bonded chips, I called you from the cleanroom on each step I wasn't 100% sure of – which may have been everything aside from QDR, I can do that like a pro – and you came straight from your office each time to help me without complaint. Thank you so much for your patience.

Pavel, thank you for always taking the time to discuss CE theory with me, from the first year when you were in the Netherlands until the very end of my project. It was very reassuring knowing that I could email you or ask for a Skype chat with any questions I had, and you would find a way to fit me into your schedule. My short visit to Prague was a real highlight of my four years and I learnt so much from you in our two day pow-wow – even

though both of our heads were aching after and I've probably only begun to scratch the surface of your knowledge.

Hans, you built my fancy little chip holder – even cloning it later, so I could do twice as many experiments in half the time! – and modified it for me on a moment's notice when I powderblasted the chip reservoirs too wide and couldn't figure out why the chip was leaking... Thank you. Jan v. N., you helped me many times with the microscopes and computers, both in the labs and personally. When I came for my interview, my laptop drive ate itself in my sleep, and you were so kind as to burn a Linux distributable for me to use... and then spent over an hour trying to save my broken portable drive a few months later (oh, technology and I).

Thank you Paul, for all your instructions in the lab, and helping me to create my own little supplies horde for my experiment marathons... and for finding the special sharpie which got away! Rik, thank you for bringing your ~~lacked~~ students to help with chem lab cleaning near the end of my project; even the false impression of having a little more time during these stressful weeks was very welcome. Loes, you helped to introduce me to conductivity detection when I first began and your thesis was a good reference for me in my early days: thank you.

Wouter, thank you for not only being my committee member for my defense, but in acting like one for the length of my entire project. I could always count on your thoughtful questions of my work in the Monday Morning presentations, and you have gone out of your way to give me positive feedback many times. Eddy, thank you for always being willing to stop and take a few minutes to answer my questions, point me in the right direction, or chat about life. Hermine, I have no idea how you manage a group of this size and still make the time to calmly help everyone with a smile. You never once turned me away from your office or asked me to come back later when I needed help with something – which I often did (and do). Thank you.

I had the pleasure of sitting in the little L-shaped-office-that-isn't-really-an-office, and despite the strangeness of its shape and location, it really became a place of warmth and comfort for me. In fact, I liked my officemates so much I even made two of them my paranympths, and had the honour of being a paranympth to another. Thank you Arpita for helping me to settle in when I first arrived, and understanding many of the struggles and emotions I had in myself the first years.

Fleur, you helped me with all the minor details of getting my setup working, digging through the lab for parts I needed, and sputtering wafers for me (I never did take that

vacuum course...) Henriette, you made our TAing for Meettechnik fun and handled so many details for us; the last year of the course was much more difficult without you. And thank you to you both for maintaining our friendship even after you left the university; our get-togethers are a source of fun and comfort for me.

Loan, we were together for almost the entirety of our PhDs, and even though our projects were so different that we couldn't really help each other scientifically, I think we buffered each other in many other ways. Thank you for your friendship, for hugging me when I needed it, for always being conscious of my feelings, and for asking me to be your paranymph. Even though you couldn't make it to my defense in person, you'll be in my thoughts.

Miguel, thank you for being my desk buddy and agreeing to be my paranymph. You supported me scientifically in my project, in the entire PhD process, and over the last two years, your friendship has been invaluable to me. It made me very happy to be able to share random daily stories with you, to distract you with kitty gifs, and to chatter companionably at our monitor screens together – you in Spanish, me in English, and us both in whatever language our other strange words belong to.

Wei-Shu, thank you for your warmth and kindness, and for agreeing to be my other paranymph. You are such a giving person, often bringing treats from your travels or Taiwan for our office, inviting me to dinner at your home, sharing your funny stories with me, and any advice you could give. I often felt you nudging me out of my shell, and your quiet encouragement and support is something I'm very grateful for.

For a time, Burcu and Wesley sat in what is now the meeting room near the printer, and since our offices were both kind of estranged from the group in a way, you both kind of became officemates by proxy for me. I felt as comfortable swinging by your offices for a chat as I felt in my own office, and I hope that you both felt the same. Wesley, thank you for always taking time to share frustrations about our projects and talk about life. It was a comfort to me that you could help Loan with all the defense day details – which you're much better at keeping on top of than I – when I had my hands full with the end of my project. Burcu, thank you for our coffee dates, for helping me with many organizational things, and for always being available when I needed someone to talk to. You've supported me in the upside-down cake my life became in the last few months, and helped me to make it fun. I look forward to seeing you standing here next year.

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brightened my mood. Zhenxia, thank you for your friendship during the short time you were at BIOS. Your warmth and encouragement brought me much comfort and support, even long after you left for home.

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Are you regretting jumping to this section first, yet? *When does it end?* There are still many members in our large group (past and present) that I have yet to mention, who I know contributed to the warm and friendly atmosphere at the University. I don't want to list everyone's name in a cold, static list, so instead I'll say: *If you're holding a copy of this book in your hands now, and are attending my defense, I'm sure that you contributed in some way – no matter how small – to this thesis being successful. Thank you!*

Robert, thank you for your support over much of the four years of this thesis and encouraging me to reach the end. You always tried to understand my frustrations with my project and were proud to explain to others what I was working on. I'm very grateful for that, and more.

In closing, I owe much to my family and friends in Canada. I've missed weddings, new children, other life events, and innumerable birthdays and holidays. When I was in Canada, people used to joke that you could never separate me from my phone; now it's a glorified mp3 player. I've been horrible at calling, even worse at writing, and practically dropped off the planet the last two years. Thank you all for still loving me and being excited to see me finish my degree (and actually *see me*) anyway.

Grandma, you are a constant light in my life and the kindest, most loving person I know. Thank you for always supporting me in all that I do. Tante Rosemary, thank you for celebrating the best in me, pointing out my downfalls, and encouraging me to get the most out of my life. Ilona, thank you for your love, understanding, and being with me through everything.

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