2019

Capillary Electrophoresis Buffer Optimization for Plant Tissue Analysis

Rebekah Davis
University of Central Florida

Capillary Electrophoresis Buffer Optimization for the Analysis of Plant Tissue

Rebekah Davis, Kaleigh Davis, Chase Mason

Department of Biology, University of Central Florida, Orlando, FL, 32817

Corresponding Author:

Rebekah Davis

(786) 210-3362

Rebekah.Davis001@knights.ucf.edu

Research Article:

Running Title: Buffer Optimization

Key Words: Capillary Electrophoresis (CE), Micellar Electrokinetic Chromatography (MEKC), Buffer, Factorial, Plant Tissue, Secondary Metabolites

Objective/Thesis Abstract

Capillary electrophoresis (CE) is an analytical chemistry approach that allows for the efficient separation by charge of diverse classes of compounds for analysis, including secondary metabolites. The goal of this work was to optimize a buffer system for plant tissue analysis using micellar electrokinetic chromatography (MEKC), and by doing so to understand the role of buffer components in the performance of this form of capillary electrophoresis. In this experiment we implemented a factorial design to optimize buffer composition for separating plant tissue and secondary metabolites. The results of this experiment will be used to optimize a universal buffer for MEKC analysis that can be used on any variety of plant tissues. To determine the feasibility of this, a diverse set of plant secondary metabolite chemical standards in solution were tested as well as *Helianthus annuus* tissue to confirm the separation in a real biological sample. The results of this optimization yield insights into the utility of buffer components like electrolyte and pH for MEKC separation.

Acknowledgements

I would like to thank Dr. Chase Mason for his assistance and guidance throughout the experiment, Kaleigh Davis for her ideas and support, and Jesse Franklin-Peiper for his help performing the experiment.

Table of Figures

Figure 1: Formula and Factors Affecting the Electroosmotic Flow…… 2

Figure 2: MEKC Capillary Cross Section …….. 3

Figure 3: Buffer Variation Across the Literature …… 4

Figure 4: Flowchart for Buffer Optimization Factorial …….. 6

Figure 5: Spreading Effects of Borate Concentration ……. 13

Figure 6: Data Tables of Peak Separation with Standard Deviations ……. 14

Figure 7: Effects of pH on MeOH and 0.5% DMSO/Water Samples …… 16

Table of Contents

Abstract …… ii

Acknowledgements …….. iii

Introduction …….. 1

Methods ……7

Results …….. 11

Discussion …… 17

Conclusion ……... 21

References …… 23

Introduction

Capillary electrophoresis (CE) is an analytical technique used to analyze the composition of a sample by separating the compounds within it based on charge and size, and allows for identification of sample makeup by passing the individual compounds passed a diode array detector yielding a UV fingerprint (Jimenez-Lozano 2002). It is a separation technique that can be used to quantify a compound and can use UV fingerprints of said compound to identify it in nearly any kind of organic or inorganic aqueous solution (Tavares 2003). CE relies heavily on electroosmotic flow (EOF) to move and separate analytes in a small capillary. The silica coating of the capillary is charged with a strong base to create a weak acid on the wall of the capillary, which is then coated with buffer to allow interaction with the capillary wall and for the electroosmotic flow to pass appropriately. Once the silica in the CE capillary has been prepared or "cleaned" by hydrolyzing it with NaOH (or another appropriate strong base) and then coated with buffer, a small quantity of sample is injected, shocked, and then allowed to separate within the bulk electroosmotic flow of the buffer. The injected buffer coats the capillary walls, creating a bilayer on the silica of positively charged electrolyte which increases the viscosity (η) of the electroosmotic flow and adds a layer of charge to the capillary wall (ε), slowing down negatively charged molecules. Sample interactions with the capillary and the buffer allow the sample components to be separated based largely on charge. The charged separation could also be affected by the pH of the buffer (ζ), which can ionize molecules in the sample and dictates how well electrolyte and sample can interact. Molecules in sample are further separated by size since large charged molecules are attracted to the electrode differently than small molecules, and as

molecules encounter surfactant, interactions with these charged micelles acting as a pseudo-stationary layer will also separate molecules by size. The negatively charged spherical aggregation of hydrophobic surfactant like SDS into amphiphilic micelles may encapsulate weakly charged or neutral species, and will separate these based on charge as well. The composition of the buffer is an important factor because it controls the electroosmotic flow (Figure 1) of molecules within the capillary, their separation, migration, and can alter the charge of compounds in sample and change the way the sample interacts with the capillary (Whatley 2001). As the electroosmotic flow flows toward the cathode, the small positively charged molecules will elute first, followed by large positively charged molecules, large negatively charged molecules, and small negatively charged molecules (Chetwynd 2018). The neutral species will be separated last by the surfactant which is most strongly attracted toward the anode. The slightly negative species will elute first, followed by the slightly positive ones which interact most strongly with the surfactant, and are therefore the furthest back (Pranaityte 2006) (Figure 2). The diode array detector (DAD) records a unique spectral signature for each molecule that passes by, and the spectra along with the peak area and the migration time of each peak can be used to determine the identity of a compound, and its concentration in the solution.

Figure 1: Formula and Factors Affecting the Electroosmotic Flow Epsilon and zeta are the two terms of this equation that were manipulated in this factorial and are directly related to the velocity of the electroosmotic flow.

Formula for the Velocity of the Electroosmotic Flow (EOF)

$$v_{EOF} = -\left(\frac{\varepsilon\zeta}{4\pi\eta}\right)E$$

ε = dielectric constant of the electrolyte
ζ = the zeta potential (volts) / a measure of the charge on the wall of the capillary
η = viscosity (Poise)

To determine the best possible buffer for plant tissue separation, it was first necessary to know the principal components of a buffer. By definition an MEKC buffer needs a surfactant to separate uncharged or weakly charged molecules and to act as a pseudo-stationary phase to separate molecules by size, electrolyte to charge the capillary wall and create opportunities for molecule interaction, and the buffer needed to be set to a specific pH to charge the sample as well as the double layer of electrolyte on the silica of the capillary (Tomasbarbarean 1995). Once the general composition of a buffer was known, dozens of articles using MEKC to separate and analyze secondary metabolites in plant tissue were assessed and categorized by their buffer compositions. The literature was found to be widely inconsistent and varied dramatically in their buffer constituents, making it difficult to adapt a system from the literature (Figure 3). Twenty papers using MEKC for secondary metabolite analysis in plant tissue were evaluated based on the levels and types of surfactant, electrolyte, and pH level used, as well as the nature of the plant tissue being analyzed, what if any additives were used in the buffer, and took note of several other relevant parameters in these model experiments. By comparing common components used to make a buffer, we identified a wide range of potential buffer combinations. A factorial design

Figure 2: MEKC Capillary Cross Section Positive molecules are white and negative ones are grey. Shows small positively charged molecules being most strongly attracted to the cathode and small negatively charged molecules most strongly attracted to the anode. Negative molecules interact with the positively charged silanol walls coated with electrolyte. Anionic surfactant like SDS is most strongly attracted to the anode and positively charged wall, and slightly positive neutral or hydrophobic species of molecules are most strongly attracted to the surfactant micelles and are therefore pulled strongly toward the anode with them.

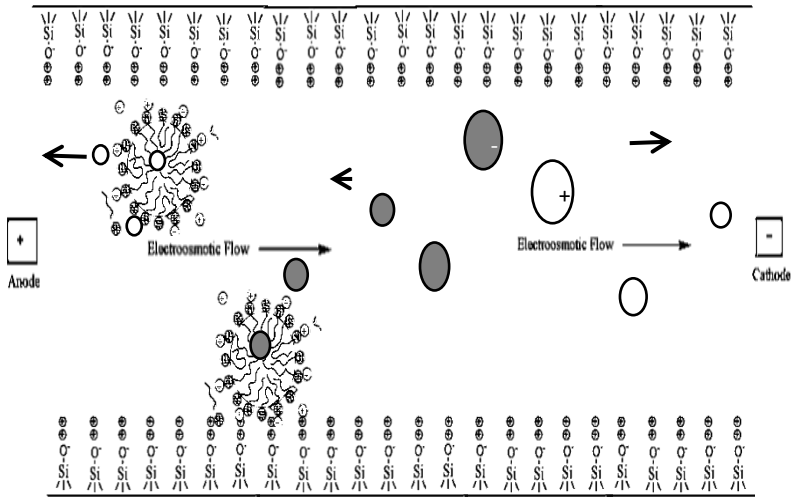
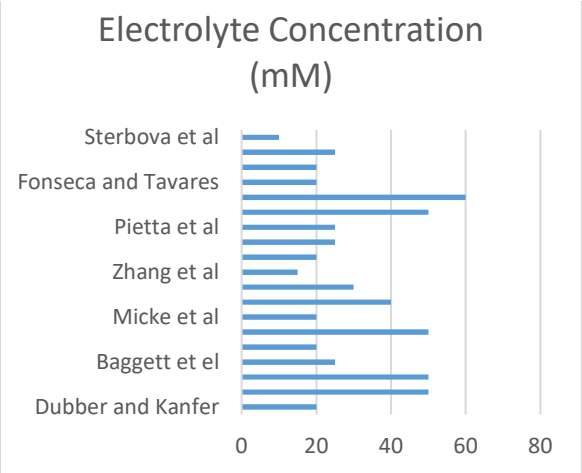
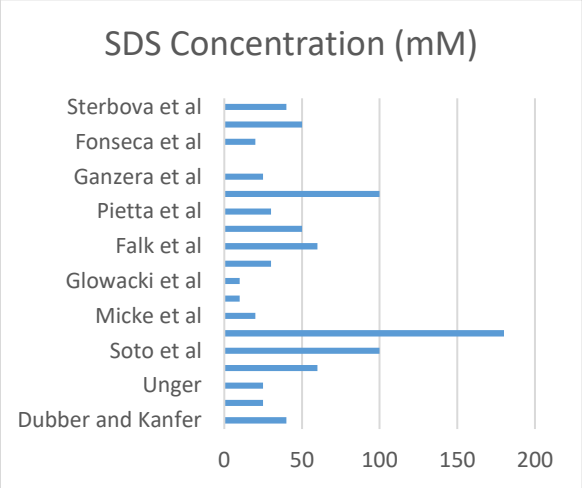
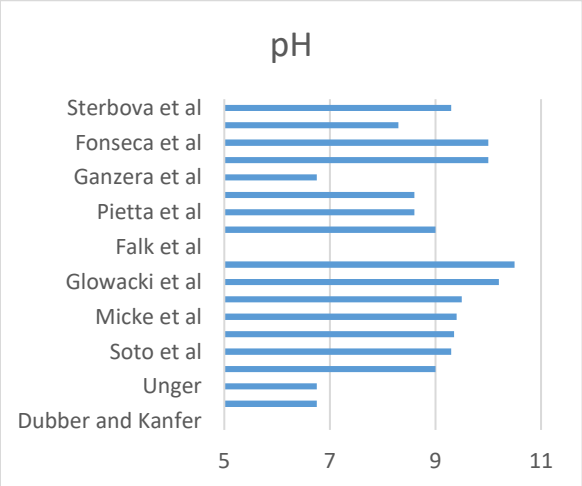


Figure 3: Buffer Variation Across the Literature Electrolyte ranged from 10 mM-60 mM, SDS ranged from 10 mM-180 mM and pH ranged from 6.75-10.5.







was used to manipulate all the different buffer components simultaneously, and this experiment focuses on the interacting charged component of the larger factorial design. Electrolyte and pH are directly responsible for the charged environment within the capillary and hold the most influence over the electroosmotic flow of molecules past the detector (Figure 1). These two interacting buffer components are the most crucial to the buffer composition and success of the separation, so these two factors were tested on the most difficult and time consuming initial step in optimizing a buffer system. A total of 40 buffers were tested at every combination of four electrolyte levels and 10 pHs as part of this charge specification factorial at 15 mM SDS. The best performing buffers will be further modified in future experiments by testing at three different surfactant levels and various levels of the two most commonly used additives. The buffers were all tested by separating a compound mix of common plant secondary metabolite standards,

and ground-truthed using real plant extracts of homogenized sunflower (*Helianthus annuus)* leaf tissue. Each of the two sample types were dissolved or extracted in two types of solvent, either 0.5% DMSO/Water or MeOH. These four total sample types were tested using all buffer combinations under the same conditions. Using the compound mix of known chemical standards, we can clearly identify the effects of each buffer component on the separation of the sample. Outlining the effects of each buffer component will assist in choosing which buffer to promote to the uncharged separation component of the factorial, and will allow a knowledgeable adjustment of future buffer composition based on understanding of how the components of the buffer are affecting sample separation.

The full factorial design tests four electrolyte levels and ten pH levels, with three surfactant levels, three levels of two different additives, and is tested using *Helianthus annuus* tissue and a compound mix of 12 different plant secondary metabolite standards, each in MeOH solvent and in 0.5%DMSO/Water (Figure 4). The factorial was broken down into three

sequential component parts, each testing the four types of sample: the Interacting Charged Component assessed here focuses on the interaction between pH and electrolyte, the Uncharged/Hydrophobic Component tests various levels of surfactant to analyze the role of size exclusion and hydrophobic analyte separation, and the third part optimizes for Increased Analyte Resolution, which is where organic modifiers like ACN and MeOH are used to sharpen peak resolution and completely separate similarly charged and structured molecules. Even though surfactant is a key component to an MEKC buffer, SDS was tested outside the initial factorial of pH and electrolyte since those two factors have a direct effect on the EOF of the buffer, while SDS does not (Whatley 2001).

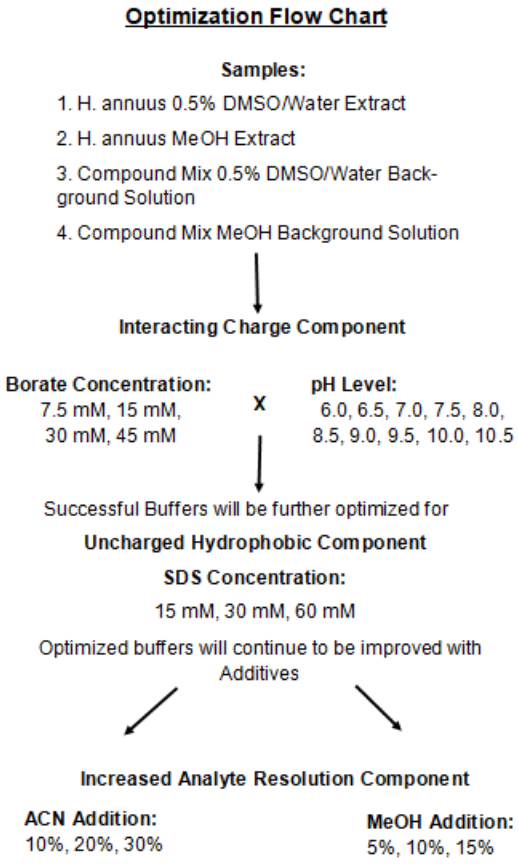


Figure 4: Flowchart for Buffer Optimization Factorial This flowchart details the full extent of the factorial to determine an optimized universal buffer for plant tissue analysis. This experiment focuses on the combination of interacting buffer components with all sample types.

Methods

Making Buffer Solutions and Extracts

To make the first set of buffers, all four levels of Sodium Tetraborate Decahydride (hereafter referred to as borate) were mixed with 15 mM Sodium Dodecyl Sulfate (SDS), both purchased standard grade from Sigma Aldrich, and were dissolved in Deionized filtered water purified through reverse osmosis. After both the borate and the SDS were fully dissolved, the final pH of each buffer was modified using phosphoric acid or sodium hydroxide to decrease or increase the pH respectively, with the assistance off a pH probe (PH-BTA, Vernier, Inc.).

. To gain an objective description of the performance and separation ability of each buffer, a mixture of known compound standards were needed. An initial 20 analytical standards of plant secondary metabolites were dissolved singly in either a methanol or a 0.5% DMSO/water solution to a final concentration of 1 mM, and these twenty standards were tested with a previously published, reasonably effective buffer to determine separation time and spectral fingerprints for each compound. These 20 compounds included flavonoids, non-flavanoid phenolics, alkaloids, and terpenes. Of these 20 compounds, a subset of 12 compounds that were identified to have sufficiently different migration times and spectral fingerprints so as to be easily identified were combined into compound mixtures with the two different solvents, methanol and the 0.5%DMSO/water solution. The compound mix contains five terpenes (myrcene, limonene, B-caryophyllene) and seven phenylpropanoids (quercetin, naringenin, catechin, coumarin, caffeic acid, salicylic acid, and gallic acid). The compound mixtures of these

common secondary metabolites were used as a simple sample matrix to test the separation abilities of the buffers and to establish a spectral library to differentiate secondary metabolites.

In addition to testing the range of buffers with the compound mixtures, the buffers were all also tested with *H. annuus* samples. This biological sample was made using finely ground, homogenized dried leaf tissue collected and pooled in equal proportion from plants of twelve inbred lines of cultivated sunflower, known as the 'core 12'. The Core 12 lines of *H.annuus* represent 50% of genetic diversity in crop sunflower, and were picked to be representative of crop sunflower as a whole (Mandel et al. 2013). Secondary metabolites were extracted from 0.05g plant tissue using either analytical grade methanol (CAS# 67-56-1, Sigma Aldrich), or with 1mL 0.5% standard grade DMSO (CAS# 67-85-5, Sigma Aldrich) and deionized filtered water. These extractions were vortexed for 30 seconds, centrifuged at 4000rpms for 10 minutes, and refrigerated for 2 days before being used as sample for analysis. After the secondary metabolites were fully extracted into the solvent, the samples were diluted 1:3 before being run in the capillary electrophoresis system.

Sequence Table and Method Breakdown

The efficacy of each buffer and sample was tested on a capillary electrophoresis (CE) system (G7100 capillary electrophoresis system, Agilent Technologies Inc.) in a capillary 56 µM wide, and 60 cm long. The conditions in the capillary were 25 ˚C with voltage at 25 kV, a current of 300 µA, and a power of 6.0 W. The Interacting Charge Component of the factorial tested the effects of pH and electrolyte, and this was done using two sequences separating the buffers by pH. The sequences were created so that the pH was slowly increased and there was minimal pH

change and disturbance of the silanol groups on the capillary wall. Significant changes to pH within a sequence affects the electroosmotic flow of the system, which can alter the migration time of the sample (Lauer 2009). To prevent ion buildup and unnecessary disturbance of the capillary wall, the buffers were tested in such a way that the pH only gradually increased between methods. When attempting to randomize pH as well as borate concentrations, Joule heating and other effects brought on by changing capillary wall conditions brought an unsteady current through the machine, and ruined the results of that sequence. To avoid that, there were two sequences run during this part of the experiment, one sequence testing the 20 buffers from 6.0-8.0pH, and the second testing the 20 buffers at 8.5-10.5 pH. Each sequence tested every buffer in those pH levels, with every borate combination, in combination with an *H. annuus* tissue extract sample from each extraction solvent, and then the sequences were repeated with the compound mixes in the same extractant solvents. The sequence is structured with repeating patterns of an 11 method intervals, starting with a cleaning method, an internal standard method, one sample type tested at all borate levels and one pH level, an internal standard, and then the other sample type was tested with the same buffers. This pattern repeats five times and increases in increments of 0.5 pH, and borate concentrations were randomized within each sequential pH level. An example of the sequence can be found in the supplement (Table S1). The cleaning method uses 600 second flushes of 1M NaOH, 0.1 M NaOH, and then triple filtered deionized water to dislodge any excess NaOH molecules not bound to the silica in the capillary, followed by a 300 second buffer flush to coat the capillary for the next sample. This cleaning run is followed by the standard 4-hydroxyacetophenone, 98% (PHAP) (CAS# 99-93-4, Sigma Aldrich), and then the sample runs. The sample runs are preconditioned with 180 second flush of

water, 0.1M NaOH, more water, and then 300 seconds of the buffer being tested to coat the newly exposed silica on the capillary walls. After preconditioning, 50 mbars of pressure was applied to the sample for 5 seconds, voltage is applied at 25 KV for 0.2 minutes, and then the matrix in the capillary is allowed to flow past the diode array for 22 minutes. The purpose of these sequences was to identify a handful of well performing buffers to test at different SDS levels and eliminate those that do not work in the system.

Buffer Selection

The sequential factorial optimization study was broken into several parts to most efficiently test all the parameters. Once the interacting charge component of the factorial with the pH and electrolyte was performed on each of the four samples, a handful of well performing buffers would be selected for further optimization. This selection process involved counting all of the true peaks from each separation and calculating the total separation time for each sample. True peaks were identified visually as an individual peak and confirmed with UV spectra. The interacting charge component of the experiment testing pH and electrolyte concentration was repeated three times so that at least three total chromatograms for every buffer with every sample were produced. This allowed us to confidently select well performing buffers for further optimization. Only peaks identified with UV spectra were counted, and the average separation capabilities of the buffers were assessed. Only buffers capable of separating more than 8 of the 12 compounds in the compound mixture and had comparable separation in the biological sample were considered to progress to the next phase of the optimization

Results

Across all sample sequences, averaged values of peak numbers and separation times between all sample runs indicated buffers in the 8.5-9.5 pH range performed the best, with some good separation at 10 and 10.5pH in conjunction with low electrolyte concentrations. Buffers at those high pH levels often experienced joule heating, or just poor separation due to the high charge conditions from a large zeta potential and the confounding epsilon potential from the heightened borate concentration. Optimal separation occurred at 9.5 pH.

A clear trend among all sequences revealed that increasing electrolyte concentration directly increases the separation of the compounds in the sample. At the higher borate concentrations, 30 mM and 45 mM, there was so much separation that some compounds were not able to migrate passed the detector before the method was terminated at 22 minutes (Figure 5). An electrolyte concentration as high as 30 mM borate might be useful when separating an unpurified or very complex solution of secondary metabolites, but more than that and the separation will take too long per sample to be an effective high-throughput analytical tool.

The compound mix of standard secondary metabolites had a cleaner separation of metabolites compared to the *H.annuus* sample. This was certainly due to its pure chemical nature and was used to clearly demonstrate the separation abilities of the buffers. Trials with these compound mix samples also had a lower standard deviation of peaks separated between sequences (Figure 6). This implies that the more purified the sample is, the easier its components will be to separate. That does not imply that a biological sample won't separate as well, but there

was more room for error, and less reliability of consistent separation between sequences with the *H. annuus* samples.

The last variable investigated in the interacting charge component of the factorial was the influence of extractant/solvent. With both the compound mixtures and the *H. annuus* samples, MeOH and 0.5%DMSO samples had an approximately equal amount of compound separation, but with some distinctive features. MeOH had slightly higher separation abilities at most pH levels, and more reliable separation between replicate runs as shown by higher peak number averages and lower standard deviations. It is important to note though that while the peak numbers and standard deviations are empirically better for MeOH, the numbers are not substantially different than the ones accumulated for 0.5% DMSO samples. The samples with 0.5% DMSO did have slightly lower separation at most pH levels, but had higher separation at the high pH levels. In addition, and perhaps most notably, the separations with 0.5% DMSO were more distinct than the ones with MeOH, with more peak resolution and a more stable baseline between peaks (Figure 7).

Figure 5: Spreading Effects of Borate Concentration Note the spreading effects of increasing electrolyte concentration. As more electrolyte is added, the more negative species take longer to migrate since they are spending more time interacting with the positive charges on the capillary wall. It is worth noting that while the migration time of the more positively charged compounds eluting around 7.5 minutes was not affected as much, adding more electrolyte did allow for the separation of four more molecules between 15 mM borate and 30 mM borate.

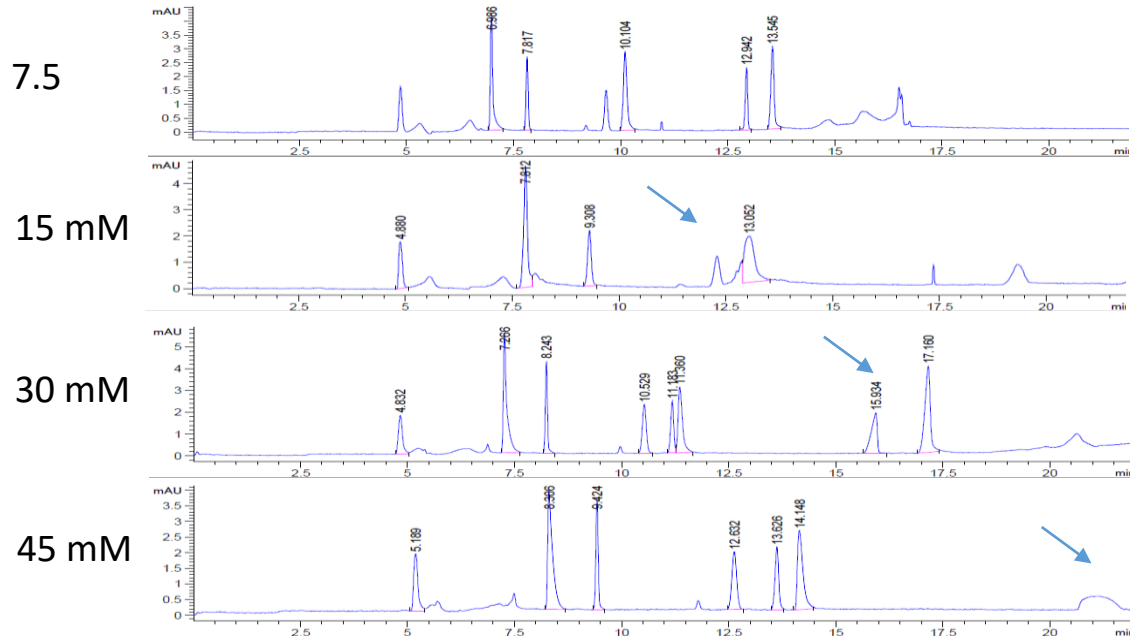


Figure 6: Data Tables of Peak Separation with Standard Deviation The charts with the number of peaks represents the average number of peaks obtained from all buffers in 3-5 runs. The standard deviations represent the standard deviation expected of the averaged peak number values based on the trials recorded.

MeOH Compound Mix Number of Peaks for Each Buffer with 15 mM SDS

		pH Levels									
		6	**6.5**	**7**	**7.5**	**8**	**8.5**	**9**	**9.5**	**10**	**10.5**
Borate Concentration	**7.5 mM**	3	2	7.4	5.8	6.7	8.333333	7	9	8.333333	7
	15 mM	2.2	2.2	5.8	6.2	6.9	9	9.5	7.5	7.666667	6.333333
	30 mM	1	2.2	5	5.4	6	6.75	6.5	7	4.333333	5
	45 mM	1.2	2.4	5.2	5	5	6.5	5	4	2.333333	3

MeOH Annuus Sample Number of Peaks for Each Buffer with 15 mM SDS

		pH Levles									
		6	**6.5**	**7**	**7.5**	**8**	**8.5**	**9**	**9.5**	**10**	**10.5**
Borate Concentration	**7.5 mM**	1.666667	3	3.5	3.666667	4	7.5	8	12	5	6.5
	15 mM	2.333333	1	3.666667	5	5.666667	5	9	12	6	8
	30 mM	1.333333	2.5	4.333333	5	1.333333	12	8.5	12.5	11	0
	45 mM	2.333333	2	4	2.666667	4.333333	10	12	6.5	2.5	0

DMSO Compound Mix Number of Peaks for Each Buffer with 15 mM SDS

		pH Levels									
		6	**6.5**	**7**	**7.5**	**8**	**8.5**	**9**	**9.5**	**10**	**10.5**
Borate	**7.5 mM**	3.666667	3.333333	5.5	5.5	6.333333	6.666667	7.333333	8	8.333333	7.666667
	15 mM	3.166667	3.333333	4.333333	5.5	5.833333	7.333333	5.666667	6.5	5.333333	8.5
	30 mM	3	3.166667	3.666667	3.666667	4.75	5.333333	5.333333	5.666667	4.666667	5
	45 mM	2.666667	2.666667	3.416667	4	4	5.333333	6.5	5.666667	4	2.5

DMSO Annuus Sample Number of Peaks for Each Buffer with 15 mM SDS

		pH Levels									
		6	**6.5**	**7**	**7.5**	**8**	**8.5**	**9**	**9.5**	**10**	**10.5**
Borate	**7.5 mM**	2.666667	2	4.666667	3.333333	4.666667	7	11.5	11.5	10	9
	15 mM	2.333333	1.5	6.333333	1.5	5.333333	8.5	11	13	8	16
	30 mM	1.666667	2.333333	3.333333	2	6.666667	10	9.5	12.5	10	4.5
	45 mM	3	2	2.333333	5.666667	4.666667	6.5	12	11	11	2

None of these peak counts include solvent front, and spectra were used to confirm the efficacy of some peaks

*some peaks did not have a smooth baseline between them, but had spectra

Standard Deviation of all MeOH Compound Mix Samples

	6	6.5	7	7.5 BAD SE	8	8.5	9	9.5	10	10.5
7.5 mM	2.12132	1.224745	1.140175	2.863564	4.494441	0.57735	1.414214	4.242641	2.516611	1
15 mM	1.30384	1.095445	0.83666	2.949576	3.847077	2	4.949747	2.12132	1.154701	1.527525
30 mM	0.707107	1.095445	2.345208	2.607681	2.915476	3.304038	4.949747	2.828427	1.154701	1
45 mM	1.095445	0.547723	2.167948	2.345208	2.44949	2.12132	2.828427	1.414214	0.57735	1

Standard Deviation for all MEOH Annuus Samples

	6	6.5	7	7.5	8	8.5	9	9.5	10	10.5
7.5 mM	1.154701	1.414214	2.12132	2.516611	1	2.12132	2.828427	0	7.071068	3.535534
15 mM	1.527525	0	2.081666	6.082763	5.507571	1.414214	2.828427	0	8.485281	5.656854
30 mM	0.57735	0.707107	2.081666	5.196152	1.154701	1.414214	4.949747	0.707107	1.414214	0
45 mM	0.57735	1.414214	3.605551	2.081666	4.163332	1.414214	1.414214	2.12132	3.535534	0

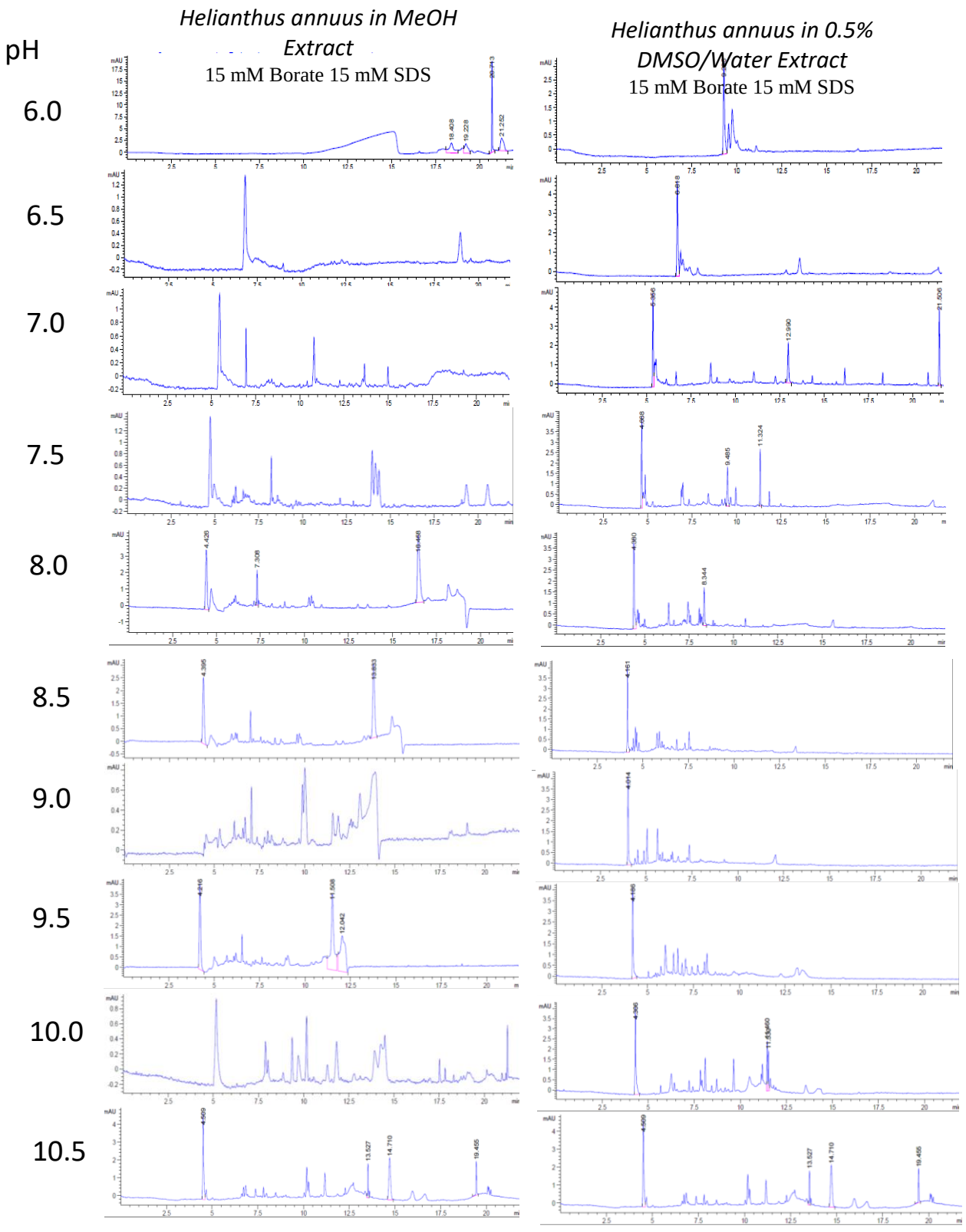
Standard Deviation of DMSO Compound Mix Samples

	6	6.5	7	7.5	8	8.5	9	9.5	10	10.5
7.5 mM	0.894427	0	0.547723	0.894427	0.547723	2.309401	1.154701	0	1.527525	3.21455
15 mM	0.447214	0	0.447214	0.547723	0.707107	0.57735	3.21455	2.12132	4.50925	2.12132
30 mM	1.516575	0.447214	0.547723	0.547723	0.83666	1.527525	2.886751	1.154701	0.57735	0
45 mM	0.83666	0.447214	0.74162	0.447214	0.447214	2.516611	2.12132	1.154701	4	3.535534

Standard Deviation for all DMSO Annuus Samples

	6	6.5	7	7.5	8	8.5	9	9.5	10	10.5
7.5 mM	1.527525	4.725816	4.725816	4.932883	4.725816	1.414214	2.12132	3.535534	2.828427	1.414214
15 mM	1.154701	3.785939	6.110101	6.082763	4.932883	0.707107	1.414214	2.828427	11.31371	1.414214
30 mM	1.154701	1.527525	3.21455	6.350853	6.429101	1.414214	0.707107	2.12132	1.414214	0.707107
45 mM	2	1	1.527525	5.507571	3.785939	0.707107	2.828427	1.414214	0	2.828427

Figure 7: Effects of pH on MeOH and 0.5% DMSO/Water Samples At low pH molecules may exhibit similar charges, and this effect is exacerbated because at low pH adsorption is suppressed. At low pH there is very little separation. As molecules begin interacting with the capillary there is more and more separation of molecules. At 9.0 pH and on there is the most separation of particles in solution, and as the pH increases, so does the ionization of those molecules. For that reason, at high pH's like 10.0 and 10.5, the charged particles are participating in more interactions and migrating further apart.


pH
Helianthus annuus in MeOH Extract
15 mM Borate 15 mM SDS
Helianthus annuus in 0.5% DMSO/Water Extract
15 mM Borate 15 mM SDS
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
10.5

Discussion

There is little consensus in the literature about what kinds of buffer to use when analyzing plant extracts with MEKC. Literature found using MEKC for this purpose varied wildly in their buffer components and component concentrations. Papers were found running analysis with buffers that spanned 10 pH levels, and exceeded the range of electrolyte and surfactant levels being tested here. Not only was there no consistency among other researchers, but none of the papers offered a reason for their choice in buffer, a critical choice to make since it dictates the capillary conditions, and the separation of analyte. Buffer compositions vary widely between labs, and in this factorial we determined what the quantitative effects of some of these differences actually are.

The electrolyte in a buffer is responsible for charging the silica on the capillary walls. The ionization creates obstacles in the capillary and can alter the local epsilon and zeta potentials on the capillary, influencing the velocity of the EOF throughout the capillary (Towns 1992). Saturating your buffer with electrolyte will hydrolyze more of the silanol groups on the capillary wall, causing the more negative molecules to bind more extensively to the wall. This results in more separation of the analyte, and an even more delayed separation for more negative species. Increasing the electrolyte concentration would be good when working with a complex sample with a wide range of molecules or when working with a lot of very negative species in solution. It's important to have a reasonable electrolyte concentration because as it increases, the charged molecules begin to interact more strongly with the capillary wall, and will get further and further apart. It's possible for molecules in the sample to still be bound to the capillary wall and still be

in the process of migrating by the time the method is done. These charged molecules might elute much later then they would have in a less electrolyte saturated solution, and so might not be captured on a chromatograph within a reasonable amount of time. For our compound mix sample, we found 7.5 mM Borate to sufficiently separate the standards at most pH levels, while in our biological sample we found that 15 mM and 30 mM Borate were better for separation.

The pH of the buffer is responsible for giving charge to the molecules in solution. At lower pH levels adsorption to the ionized silica walls is suppressed, so there are less interactions with the capillary wall and less differentiation of molecules (Towns 1992). At higher pH's when there are more free hydronium ions in solution the particles in the sample are expected to become partially positively charged. This variation in charge allows the molecules to become more separated from each other in the capillary, or closer together when they share the same charges. Ionizing the molecules in the sample changes the migration time of those molecules (Jones and Jandik 1991), making pH a critical component of an MEKC buffer. This is demonstrated in Figure 7 where the only difference between each chromatogram is the pH, and thus the charge of the compounds in solution. At pH 6.0 and 6.5 the sample is very widely spread out and nearly unrecognizable as individual compounds. The molecules become widely dispersed at pH 7, and then move closer to each other and seem to display a more uniform charge from 7.5-9.0. At 9.5-10.5 some of the molecules have become more charged and separated. This upper range of pH levels performed better than the other ranges of pH since it allowed for more variation of charges between molecules and a clean separation of molecules in a timely manner. Within the upper range of pH, 9.5 gave on average the most separation of compounds in the compound mix and in the biological sample. While 10 and 10.5 pH gave good separation, they often lead to Joule

heating which were presented as power/current issues in the capillary when the electrolyte was increased, giving a wildly unsteady baseline in the chromatograms and often makes it impossible to identify any separation of compounds.

The last variable tested in this factorial was the effect of sample extractant on the separation abilities of that sample. Every combination of buffers was tested in parallel on each of these two types of samples, either 0.5% DMSO/Water or methanol. Both of these extractants proved to be a good solvent for secondary metabolites. On average methanol extracts were able to separate slightly more compounds than the DMSO samples, but they had less clarity. Peaks were distinct, and compounds were still able to be identified using spectral signatures, but the baselines were flatter and the compound peaks were more resolved in the DMSO runs. It's possible that with more replications the average number of peaks separated with each solvent would be more similar and they would prove to be more reliable with a smaller standard deviation of peaks separated between sequences. There were only three replicates performed of each sample with each buffer combination, and outliers collected could be influencing the averages and standard deviation between sequences. However, the qualitative appearance of the chromatograms suggests that alcohol-based extracts may have lower reproducibility in MEKC.

Moving forward in the larger optimization study, buffers at 8.5, 9.5 and 10.5 will be tested with 7.5 mM and 15 mM Borate, as well as at 15 mM, 30 mM, and 60 mM SDS. In this uncharged separation component of the buffer optimization, the best concentration of pseudo stationary phase will be tested for optimal separation of molecules by size, as well as to understand how well the buffer can separate uncharged or hydrophobic molecules. After that, no more than 5 total buffers with optimized pH, electrolyte, and surfactant will be used to analyze

all sample types while testing the different additives and concentrations of additives. Once a high performing optimized buffer is selected, it will be used for analysis with 100 species of plants from all major families and classes ranging from ferns to temperate trees. Should the separations be successful, our buffer would be a universal starting place for plant tissue analysis using MEKC capillary electrophoresis.

Conclusion

Buffers at 9.5 are optimal for secondary metabolite separation, with good separation also seen at pH's 8.5-10.5. Electrolyte concentration and pH are critical components for compound separation and for controlling the charge within a capillary. Simple samples can be effectively separated with electrolyte concentrations as low as 7.5 mM borate, and more complex samples can be cleanly separated at borate concentrations between 15 mM and 30 mM. CE is a robust analytical separation tool capable of separating components of sample regardless of sample solvent, but 0.5% DMSO solvents yield sharper peaks and a flatter baseline compared to samples using methanol as the solvent. Total recovery of samples is not always possible, but efficient, effective, high-throughput analysis is achievable with capillary electrophoresis.

Author Contributions (no order within, alphabetical)

Initially conceived and designed the study:	Kaleigh Davis, Chase Mason
Subsequent study design contributions:	Kaleigh Davis, Rebekah Davis, Chase Mason
Primary data collection:	Rebekah Davis
Manuscript statistical analysis:	Rebekah Davis
Manuscript figure making:	Rebekah Davis
Initial manuscript writing:	Rebekah Davis
Manuscript editing/feedback	Kaleigh Davis, Rebekah Davis, Chase Mason

References

1. Alagar et al. (2014) Updated Review on Micellar Electro kinetic Chromatography. *Chromatography Separation Techniques, 5:3 doi.org/10.4172/2157-7064.1000231*
2. Chetwynd et al. (2018) Current Application of Capillary Elecrophresis in Nanomaterial Characterization and its Potential to Characterize the Protein and Small Molecule Corona. *Nanomaterials* v8(2) 99 doi: 10.3390/nano8020099
3. Jimenez-Lozano et al. (2002) Determinination of pKa Values of Quinolones from Mobility and Spectroscopic Data Obtained by Capillary Electrophoresis and a Diode Array Detector. *Analytica Chimica Acta* 464, 37-45
4. Jones, W. and Jandik, P. (1991) Controlled Changes of Selectivity in the Separation of Ions by Capillary Electrophoresis
5. Lauer, H. Rozing,G. (2009) High Performance Capillary Electrophoresis: A Primer. *Agilent Technologies*
6. Mandel et al. (2013) Association Mapping of the Genetic Consequences of Sunflower Selection. PLoS Genet 9(3): e1003378. doi:10.1371/journal.pgen.1003378
7. Pranaityte and Pandarauskas (2006) Characterization of the SDS-induced electroosmotic flow in micellar electrokinetic chromatography with cationic polyelectrolyte-coated capillaries. *Electrophoresis, 27, 1915-1921*
8. Tavares et al. (2003) Applications of Capillary Electrophoresis to the Analysis of Compounds of Clinical, Forensic, cosmetological, Environmental, Nutritional,

and Pharmaceutical Importance. *Journal of the Brazilian Chemical Society*, 14 (2) http://dx.doi.org/10.1590/S0103-50532003000200016

9. Tomas- Barberan, A. (1995) Capillary Electrophoresis: A New Technique in the Analysis of Plant Secondary Metabolites. *Phytochemical Analysis*, 4, 177-192
10. Towns, Regnier (1992) Impact of Polycation Adsorption on Efficiency and Electroosmotically Driven Transport in Capillary Electrophoresis. *Analytical Chemistry, 64, 2473-2478*
11. Whatley, Harry (2001) Basic Principles and Modes of Capillary Electrophoresis. *Clinical and Forensic Applications of Capillary Electrophoresis*, 21-58.
12. https://chem.libretexts.org/Bookshelves/Analytical_Chemistry/Supplemental_Modules_(Analytical_Chemistry)/Instrumental_Analysis/Capillary_Electrophoresis