Key Distance Learning Module I: Getting to Know Your Capillary Electrophoresis System

C.M. White, K.M. Hanson, L.A. Holland*, C. Eugene Bennett Department of Chemistry, West Virginia University, Morgantown, WV 26505

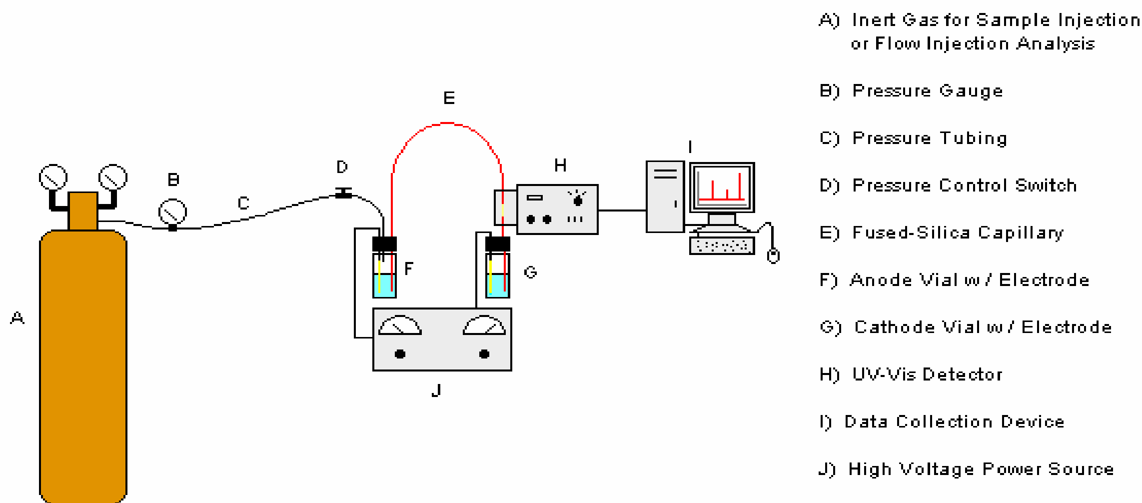
*corresponding author: Lisa.Holland@mail.wvu.edu, 304.293.3060 x6214
This work is licensed under the Creative Commons Attribution-Non Commercial-Share Alike License
Return to Distance CE Homepage
Return to the Learning Module Homepage

Alternate Materials

The buffer, tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), and analytes, atenolol, mesityl oxide, tolmetin may be replaced. Ideally, the lab should include one positively charged analyte, one negatively charged analyte and a neutral marker in order to demonstrate migration order experimentally. The cation and anion we chose to work with maintained charge at pH 7. We selected TES as the running buffer because it buffers at pH 7 and provides a low background current. You may choose other systems, for example riboflavin (net neutral charge at pH7), ascorbic acid (negative charge at pH 7), and thiamine (net positive charge at pH 7). In this case phosphate buffered at pH 7, may be used as a background electrolyte. Before asking students to use different chemicals, we recommend you perform the experiments outlined in Learning Module I with your substitute set.

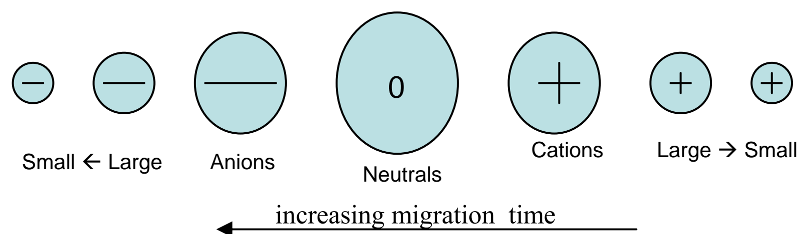
Procedures: The Module

1. Draw a schematic representation of a capillary electrophoresis system. Include instrumental components. Make the diagram as detailed as possible.



This material is based upon work supported by the National Science Foundation under Grant No. 0307245.

2. How are analytes separated by free zone capillary electrophoresis? Describe the fundamental principles that govern the method. Movement within the capillary is dictated by the electrophoretic mobility and electroosmotic flow, and net migration is the sum of both of these contributions to motion. Typically, injections are performed at the anode, which is positively charged, and detection is achieved at the cathode, which is negatively charged. Bodies of opposite charge are attracted to each other (and conversely, those of similar charge are repelled). The electrophoretic mobility of cations is towards the cathode and that of anions is towards the anode. Large molecules have greater frictional drag than small molecules, and electrophoretic mobility is based on charge-to-size ratio. The electroosmotic transport is the bulk flow generated by the movement of the buffered solution in the electric field induced at the charged surface of the capillary wall. The figure below summarizes the order of migration in a typical capillary electrophoresis system (injection end at anode, detection end at cathode, bare fused silica capillary, pH > 5).



3. What type of detector will you use for this research project? What fundamental spectrophotometry principle(s) govern(s) analyte response in the detector? The analyte migration through the capillary will be detected via a UV-visible absorbance spectrophotometer. Light of a specific wavelength is focused using an optical lens through a window that is burned through the polyimide coating on the capillary. The ratio between the light coming out of the capillary window and the light going in the capillary is the transmittance. The absorbance, which is used when quantifying analytes, is the log of the transmittance value, and is related to analyte concentration by Beer's Law. If the absorbance value of an analyte is greater than the buffering solution at a specific wavelength, a peak will be generated on an electropherogram for the time period during which the analyte passes through the detection window.

4. Define the following terms: quantitative, qualitative, mean, median, standard deviation, relative standard deviation, significant figure, linear range, dynamic range, reproducibility, limit of detection, limit of quantification, analyte, sample, standard, and calibration curve.

   Note: there are many excellent resources that contain a more thorough discussion of the terms defined below. One example is D.C. Harris, Quantitative Chemical Analysis 6th edition, W.H. Freeman and Co., NY, 2003.

   Quantitative relates to numerical determination of an analyte, in other words, how much is in the sample.

   Qualitative pertains to the composition of a sample, in other words, what is in the sample.

   Mean is the average value of a data set.

   Median is the value at the midpoint of a data set. This can be determined by arranging the data in ascending or descending order.

This material is based upon work supported by the National Science Foundation under Grant No. 0307245.

Standard Deviation is a measure of the deviation of values in a data set from the mean of these values. Consult a quantitative analysis textbook, such as that by D.C. Harris cited above, for the formula.

Relative Standard Deviation is the ratio of the standard deviation (defined above) and mean value (RSD = SD/mean.

Significant Figures are the digits in a numerical measurement that are known with a degree of certainty in the measurement. By convention, the last reported figure contains some uncertainty.

Linear Range is the range of values over which the relationship between analyte concentration and analyte signal can be defined by a straight line (linear) with a specified error in this fit. This range may be used for quantitation.

Dynamic Range is the maximum range over which the analyte signal can be related to concentration. Note, the relationship between analyte concentration and analyte signal is not necessarily linear.

Reproducibility is a measure of the precision associated with the analytical measurement.

Limit of Detection is the minimum analyte concentration at which the analyte response, or signal, can be distinguished from the noise. The quantitative definition of this term varies, and is frequently identified or specified along with the reported value.

Limit of Quantification is the minimum analyte concentration than can be quantified.

Analyte is the compound or element being quantified or reported.

Sample contains analyte in some matrix, known or unknown that is analyzed by the instrumental or analytical method.

Standard contains a known concentration of analyte, in some matrix, used as a reference for quantitation.

Calibration Curve is a graph of the analyte signal (Y-axis) and the analyte concentration (X-axis) for a given analyte and analytical procedure.

5. Write the standard operating procedure to make a stock solution of 1mM tolmetin dissolved in deionized water. Include a sample calculation.
   # of moles of tolmetin in 10 mL of 1.0 mM stock: 0.010 L x 1.0 mM = 0.000010 moles tolmetin
   grams of tolmetin in 10 mL of 1.0 mM stock: 0.000010 moles tolmetin x 315.3 g/mol = 3.1_5 mg tolmetin

   NOTE: a standard operating procedure is unique to each lab. Below is one example.

   A) Consult the MSDS found in the MSDS lab record for the analyte you are working with if you have not done so previously. Log all information in your laboratory notebook.

   B) Inspect the balance prior to use. Log all pertinent information into the balance log book (condition, analyte, balance calibration, date, time, analyte quantity). Obtain a clean piece of weigh paper and a small spatula. Using clean tweezers fold the weigh paper in half. Still using the tweezers, place the paper on the balance pan. With the weigh paper on the balance pan, tare the balance. The balance pan should now read 0.00000g. Using the clean spatula, add tolmetin to the weigh paper

This material is based upon work supported by the National Science Foundation under Grant No. 0307245.

to obtain a reading of ~0.0032 g. Record the reading. Using clean tweezers, transfer the tolmetin to a clean 10-mL volumetric flask that has been previously calibrated. Using the tweezers, place the weigh paper back on the balance pan. Record the reading. The difference between the two readings is the amount of tolmetin in the 10-mL volumetric flask.

C) Add the appropriate volume of diluent to a calibrated volumetric flask to the mark. The flask must have been calibrated previously to the mark. Avoid parallax by viewing the fill mark at eye level. The bottom of the meniscus should be flush with the mark as was the case in the calibration of the flask. If the mass of tolmetin is not 3.2 mg as in the above calculation, or the flask volume is not 10-mL exactly, calculate the stock concentration as appropriate.

D) Replace the cap on the flask and thoroughly mix the solution until the stock solution is dissolved.

E) Transfer the contents of the flask to a container appropriate for storage, such as a clean 15-mL centrifuge tube. Protect the stock from light by covering the centrifuge tube w/ aluminum foil and store in freezer until needed.

6. Write the standard operating procedure to dilute a 1 mM stock solution of aqueous tolmetin to a final concentration of 100.μM. Note: the 100.μM tolmetin must be diluted in 50mM pH 7.0 N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES). Include a sample calculation.

$M_1V_1 = M_2V_2$ $\qquad$ $M_1 = 1.00 \times 10^{-3}$ M, $V_1 = ?$, $M_2 = 1.00 \times 10^{-4}$ M, $V_2 = 3.00 \times 10^{-3}$ L

$\therefore V_1 = 0.300$ mL

Procedures for diluting 0.300 mL 1 mM tolmetin stock with 2.70 mL TES outlined below.

1) Retrieve tolmetin and 50 mM pH 7.0 TES stock solutions* and allow to equilibrate to room temperature.
2) To a clean sample vial, add 0.300 mL tolmetin with a micropipette. Change pipette tips. Add 2.70 mL TES to the sample vial to bring the total volume to 3.00 mL.
3) Mix by inversion or vortexing.

* Consult supplementary standard operating procedures for protocols for making stock solutions.

7. Mesityl oxide and tolmetin were separated using free zone electrophoresis under the following conditions: 50 micron i.d., 42 cm fused silica capillary, 50 mM pH 7.0 phosphate buffer, separation voltage 16kV, detection at 206 nm. Using the information provided below, report the mean migration time, mean peak height, mean peak area, mean plate count/meter for mesityl oxide as well as tolmetin. For each mean value determined, also report the standard deviation and relative standard deviation.

Run #	Analyte	Migration Time	Peak Width at Base	Peak Height	Peak Area
1	mesityl oxide	3.682	0.10	21932	59390
	tolmetin	7.975	0.19	11295	99031
2	mesityl oxide	3.758	0.11	22841	65364
	tolmetin	8.175	0.20	13255	110354
3	mesityl oxide	3.725	0.10	22780	63392
	tolmetin	8.329	0.20	12326	105155

This material is based upon work supported by the National Science Foundation under Grant No. 0307245.

<u>Mesityl Oxide</u>
Mean Migration Time in min = 3.72_2 (SD = 0.03_8, RSD = 1 %)
Mean Peak Height = $2.25_2 \times 10^4$ (SD = $5._1 \times 10^2$, RSD 2 %)
Mean Peak Area = $6.2_7 \times 10^4$ (SD = $3._0 \times 10^3$, RSD = 5 %)
Mean Plate Count = $2.0_8 \times 10^4$ (SD = $1._9 \times 10^3$, RSD = 9 %)
Mean Plate Count/Meter = $4.9_6 \times 10^4$ (SD = $4._5 \times 10^3$, RSD = 9 %)
<u>Tolmetin</u>
Mean Migration Time in min = 8.1_6 (SD = 0.1_8, RSD = 2 %)
Mean Peak Height = $1.22_9 \times 10^4$ (SD = $9._8 \times 10^2$, RSD = 8 %)
Mean Peak Area = $1.04_8 \times 10^5$ (SD = $5._7 \times 10^3$, RSD = 5 %)
Mean Plate Count = $2.75_6 \times 10^4$ (SD = $7._5 \times 10^2$, RSD = 3 %)
Mean Plate Count/Meter = $6.5_6 \times 10^4$ (SD = $1._8 \times 10^3$, RSD = 3 %)

8. List the compounds of interest for the first phase of research. Compounds of interested for this lab are an analyte that is positively charged at pH 7, such as atenolol, a neutral marker, such as mesityl oxide or dimethylformamide, and an analyte that is negatively charged at pH 7, such as tolmetin.

9. By what mechanism(s) do the compounds comprising the answer to question 8 separate via capillary electrophoresis? Atenolol and tolmetin separate according to their charge-to-size ratio (electrophoretic mobility) in an aqueous solution at pH 7.0. While the sizes of the individual molecules may vary, NSAIDs will have a net negative charge at pH 7.0 and the β-blockers will have a net positive charge at pH 7.0.

10. You are given three samples to analyze using a capillary electrophoresis-UV-visible absorbance system. Sample A contains ascorbic acid and mesityl oxide, sample B contains tolmetin and mesityl oxide, sample C contains mesityl oxide and either ascorbic acid or tolmetin. All analyte are at a concentration of 100 micromolar TES buffered at pH 7. Outline the procedure you would use to analyze these samples with your instrument and determine the identity of the analyte peaks in sample C. Assume you have access to standards if necessary. Provide rational for your outlined procedures. Note any anticipated difficulties you might encounter.
Sample A: 100. µM tolmetin and 100. µM mesityl oxide in TES
Sample B: 100. µM ascorbic acid and 100. µM mesityl oxide in TES
Sample C: 100. µM mesityl oxide, 100. µM analyte (tolmetin or ascorbic acid)

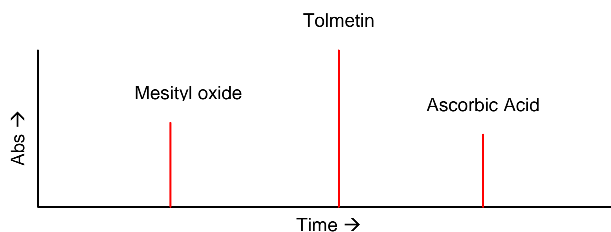
    1) The standard operating procedure for the custom-built CE system outlines procedures for initiating CE runs. First the capillary is flushed with 1M sodium hydroxide, deionized water, and 50 mM pH 7.0 TES buffer.

    2) Samples A and B are each injected a using a 2 second injection at 2 psi, and separated using an applied voltage of 10kV. Ascorbic acid is approximately ½ the formula weight of tolmetin and should migrate after tolmetin. The expected order of migration is mesityl oxide (EOF Marker) and then either tolmetin followed by ascorbic acid. Multiple trials of samples A and B need to be performed to find the mean values of migration time, and the peak dimensions. The purpose of these runs is to establish the order of migration for an NSAID and ascorbic acid. Keep in mind the mesityl oxide (neutral marker) should have the same or similar migration time in samples A or B.

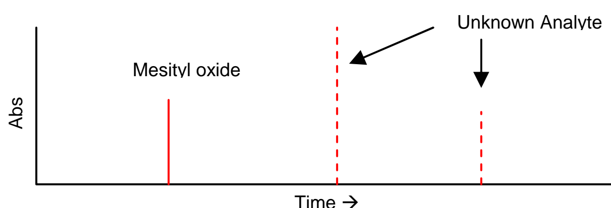
    3) Sample C containing unknown is analyzed under the same parameters.

    4) A comparison of the electropherograms for samples A, B and C can aid in analyte identification of sample C. See figure below:

This material is based upon work supported by the National Science Foundation under Grant No. 0307245.



The electropherograms of Samples A and B show the migration times of the three analytes.



The electropherogram of Sample C will show the peak for mesityl oxide, and peak in the region where tolmetin appeared, or in the region where ascorbic acid appeared.

11. Write a standard operating procedure for entering information into your laboratory notebook.

A) Start each entry on a clean page. Record the project title and the date at the top of the page.

B) Each entry consists of the following sections: Project Summary, Sample Prep, Analytical Protocol, Data, Conclusions, and Future Directions.

Project Summary—brief description of the experiments for that day.

Sample Prep—information about the samples to be analyzed during the day (for example solvent, necessary calculations, and preparation protocol).

Protocol—contains an outline of the analytical procedures being performed.

Data—charts, tables, electropherograms, graphs, containing information from the day's experiments. Also includes a listing of where data can be found if it is stored in a computer.

Conclusion—an interpretation of results. Comment on any sources of deviation from anticipated results, or the effects of any changes in the standard operating procedure.

Future Directions—statement about the next research step.

C) Once the entry is completed, the bottom of the page should be signed and dated. If an entry spans multiple pages, then the next page should be indicated at the bottom of each page, and the preceding page should be indicated at the top of each page.

This material is based upon work supported by the National Science Foundation under Grant No. 0307245.

12. You are trying to separate a sample that contains both mesityl oxide and ascorbic acid. What is the order of migration of each analyte? (i.e. which analyte comes off the capillary first?) Explain/justify your answer. Mesityl oxide migrates first, followed by ascorbic acid. Under conventional capillary electrophoresis conditions the order of migration is cations, neutrals, and then anions. At pH 7.0, mesityl oxide is a neutral compound. Ascorbic acid is anionic (pK_{a1} = 4.6, pK_{a2} = 11.6). Therefore mesityl oxide should migrate first and ascorbic acid should migrate second.

13. How do you determine the identity of analyte peaks in an electropherogram? One method for peak identification is based on the order of migration and the use of standards. By knowing the number of analytes in a sample, their relative sizes, and their net charges at the analysis pH, a rough order of migration can be established. The order can be confirmed by using a series of standards containing an EOF marker and one of the unknown analytes. Another method for peak identification is to spike an aliquot of the analyte sample with a known quantity of one of the analytes and then see which peak becomes larger. Spiking different aliquots with known quantities of a single analyte can aid in identifying peaks. Neither of these methods work with an data obtained from flow injection analysis.

14. How do you determine the concentration of analyte in a sample using CE-UV-visible absorbance detection (assume no sample matrix effects)? To quantify the concentration of an analyte using CE-UV-visible absorbance detection, the response factor of the analyte signal must be determined. Often quantitative assessment is accomplished with a calibration curve constructed from standard solutions of that analyte in the same matrix, with the same detection wavelength and separation parameters.

15. Why is flushing/rinsing the capillary important? What flush/rinse procedures should you use? The inner wall of fused-silica capillary is negatively charged when the running buffer pH is > ~4.5. These charges are critical to inducing the electroosmotic flow in the presence of the electric field. Flushing the capillary keeps the inner surface charged and free of absorbed analyte and refreshes the inner surface of the fused silica capillary. This helps to sustain reproducible electroosmotic flow and separation efficiency by reducing non-specific surface interaction induced by adsorbed species. The capillary should be flushed with sodium hydroxide, followed by deionized water and then running buffer.

16. What safety hazards must you consider before beginning this project? The safety hazards include working with high voltages, working with aqueous solutions, and physiologically-active analytes. Manipulating the capillary poses a hazard if the capillary shatters. As a result, protective eyewear, a lab jacket, and disposable gloves should always be worn when conducting experiments.

17. Write the standard operating procedure to analyze the samples listed below using flow injection analysis with your CE-UV-visible absorbance instrument. Analytes: mesityl oxide, tolmetin, atenolol. Flow injection analysis does not introduce a separation mechanism. It is useful only to check that the detector is working properly before beginning the capillary electrophoresis runs, which require more time. Since all analytes elute from the capillary together, flow injection analysis produces minimal information about the nature of the analyte present.

18. What is the molecular weight of each analyte listed below? Analytes: mesityl oxide, tolmetin, atenolol.
mesityl oxide – 98.9 g/mol, tolmetin – 315.3 g/mol, atenolol – 266.3 g/mol

19. What is the net charge of each analyte in aqueous solution buffered at pH 7.0? Analytes: mesityl oxide, tolmetin, atenolol. Mesityl oxide is neutral (net charge is 0). Tolmetin is anionic (net charge is -1, pK_a ~ 3.5). Atenolol is cationic (net charge is +1, pK_a ~ 9.6).

This material is based upon work supported by the National Science Foundation under Grant No. 0307245.

20. In a conventional free zone capillary electrophoresis system (i.e. anode at injection end, cathode at detection end), what is the order of migration of the following analytes: mesityl oxide, tolmetin, atenolol (assume background electrolyte is aqueous, buffered at pH 7.0)?
    1) atenolol, 2) mesityl oxide, 3) tolmetin

21. Write the standard operating procedure to analyze the sample listed below using free zone capillary electrophoresis with your CE-UV-visible absorbance instrument. Be sure to include protocol for injection, detector operation, and flushing/rinse. Sample contains the following analytes: mesityl oxide, tolmetin, atenolol.
    There should be at least three samples prepared. The first contains mesityl oxide and tolmetin, the second contains mesityl oxide and atenolol, and the third sample contains all three analytes. If you have no idea what the detector response is for any given concentration for that analyte, you may opt to make three samples, each containing only a single analyte. The single component samples can be quickly analyzed using flow injection analysis to confirm analyte presence, and the best range setting. The former three samples can be used to establish the order of migration. The sample containing all three analytes is the target sample, and analysis should be performed in triplicate to obtain usable date. When analyzing data, the standard deviation and the relative standard deviation should be calculated for the migration time, peak height, and mean plate count.

22. Provide an electropherogram of a single sample containing the following three analytes: neutral marker (mesityl oxide or dimethylformamide), tolmetin, atenolol, each at a concentration of 100 micromolar. Note, this solution must be diluted with background electrolyte. Identify each peak in the electropherogram. Indicate how you verified the identity of each peak experimentally. This will vary depending upon the separation conditions and procedures the student has used.

23. Using the electropherogram provided for question 22, complete the following information:

sample: 100 µM tolmetin + 0.01% DMF + 200 µM atenolol	run date and time: March 17, 2004, 3:46 pm
run name: CZE 20kV 200uMAT 100uMTol 0.01%DMF third trial	computer filename: CESamp3Run1
notebook entries: Christian #2, page 56	capillary dimensions: 36cm long, 50micron ID, window 8 cm from cathode (detection end)
background electrolyte: 25 micromolar CHES pH 9.3	
flushing protocol: Immediately before trial run, capillary flushed 30 minutes with filtered 1N NaOH, 15 minutes deionized water, 30 minutes CHES pH9.3	
injection duration: 1.5 seconds	injection pressure: 5 psi
separation voltage: 20 kV	separation current: 0.009 mA
detection wavelength: 206 nm	detection range: 0.0005 mAU full scale
detection rise time: 0.3 second	
tolmetin migration time: 147.404 seconds	tolmetin peak height: 0.214909
tolmetin width (σ) 1.7152	tolmetin plate count: 7386
neutral marker migration time: 705.103	neutral marker peak height: 0.862209
neutral marker width (σ) 1.68518	neutral marker plate count: 3890
atenolol migration time: 92.471 seconds	atenolol peak height: 0.12498
atenolol width (σ) 1.9974	atenolol plate count: 2143

This material is based upon work supported by the National Science Foundation under Grant No. 0307245.