THE CHEMICAL EDUCATOR
ISSN 1430-4171
http://journals.springer-ny.com/chedr
S 1430-4171 (97)06075-5

Appendix B: Sample Student Protocol

Learning to Learn:

Introduction to Capillary

Electrophoresis

Protocol for Determination of Caffeine in

Soda Pop

Using the Waters Quanta 4000E

ISSN 1430-4171
http://journals.springer-ny.com/chedr
S 1430-4171 (97)06075-5

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Analytical Chemistry 1231

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February 24,1996

ISSN 1430-4171
http://journals.springer-ny.com/chedr
S 1430-4171 (97)06075-5

Protocol for Determination of Caffeine in Soda Pop:

Using the Waters Quanta 4000 E Capillary Electrophoresis System

INTRODUCTION

ABSTRACT-

In this experiment we will "learn how to learn" about new instrumentation. We will learn about *Capillary Electrophoresis* through reading the manual for the Waters Quanta 4000 E Capillary Electrophoresis System Operator's Manual, and performing a simple test experiment using a known caffeine standard. We will then apply the skills acquired in processing a known sample, to determination of the quantity of caffeine in an unknown soda sample.

MATERIALS AND METHODS(1)-

The Waters Quanta 4000E performs electrophoretic separation, UV-vis detection and fraction collection.

Electrophoresis separates charged analytes on the basis of their differential migration toward an electrode through a moving electrolyte. A power supply within the system creates a voltage potential across a capillary. Components of the analyte travel along the capillary toward the cathode and collection vial. Flow of the electrolyte causes all molecules to move toward the cathode. Cations travel more quickly than anions which have an affinity for the anode.

The time it takes for a compound to travel across a given length of capillary at a given voltage is characteristic of that compound. The Waters 4000 E measures the migration time of an analyte from the time

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voltage is applied to the system to the time the analyte reaches the UV-vis detector.

The specific wavelength detected by the UV-vis detector can be selected by changing the filter and lamp in the optics bench. Caffeine has a π-π^* chromophore with an absorption band that can be detected within the normal operating wavelengths of the Waters 4000E, using the zinc lamp with the standard 214 filter (2).

When the Waters 4000E is linked to a chart recorder, an electropherogram can be produced. The electropherogram is a chart that plots time versus voltage. The location of a peak along the x-axis indicates the time at which a compound passes the UV-vis detector. This represents a compound's migration time and can be used to identify the compound by comparison to a known standard. The intensity of the peak corresponds to the quantity of the analyte in the sample, and can be used to quantify the analyte.

In the first part of this experiment we ran a known caffeine sample and compared our results to known data standards for that sample. In the second part of this experiment, outlined in the Procedure section of this report, we will attempt to quantify an unknown amount of caffeine in a sample of soda pop using the Waters 4000E.

I found three ways to quantify the amount of caffeine in the soda sample. The *Method of Standard Addition* has been used to quantify caffeine in urine (3). A *Calibration Curve* has been used to compare HPLC peaks of caffeine concentration in the brain (4). Finally, brain-storming with fellow students led to the conclusion that Beer's Law could be used to determine the unknown concentration.

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PROCEDURE

PREPARATION OF REAGENTS-

Buffer-

Prepare 100 mL of 50 mM, pH 7 phosphate buffer solution to be used as the electrolyte and for purging the capillary as follows:

*Pick a form of acid based on the desired pH. KH_2P0_4 has a $K_2 = 6.17 \times 10^{-8}$ So with a pH = 7.21 it is a good choice.

*Determine the number of grams of salt needed for the solution:

$0.1L.* (136.1\ g/mole)*(5.0 \times 10^{-2}\ moles/L) = 0.6805\ g.\ KH_2PO_4$

*Place the calculated amount of salt in a flask with a mouth large enough to accommodate a pH meter, such as an Erlenmeyer or beaker

*Measure 100 mL of distilled water, add most of it to the flask, swirl well to mix

*Measure the pH

*Add concentrated NaOH drop wise while mixing until pH 7 is reached

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*If you overshoot a little, carefully add a slightly diluted strong acid such as HCl to bring back to pH 7 note- always add acid to water

*When pH 7 is reached fill to 100 mL with distilled water

Degassing and Filtering Buffer-

Gas in the buffer solution can have numerous negative side effects on Capillary Electrophoresis. Degassing is **essential** to ensure accurate reproducible results. Impurities in the buffer can lodge in the capillary and prevent proper flow, filtering prevents this occurrence.

*Degassing and filtering can be accomplished by pouring the buffer through an HPLC filter (0.45 μm is suggested) into a water aspiration flask

*After the buffer filters through, vigorously swirl the flask containing the buffer while maintaining the vacuum aspiration for approximately 20 minutes

Caffeine Standard Solutions-

Preparation will depend on the method chosen:

*Standard Addition- Prepare 1 mL solutions of caffeine with concentrations of 1, 2, 3 and 4 mg/mL.

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*Calibration Curve- Prepare four l mg/mL solutions of caffeine, dilute the first to 15 mL, the second to 10 mL, the third to 5 mL, and leave the third as initially prepared

*Beer's Law- Prepare a 1 mg/mL solution of caffeine

Soda Samples-

*Allow the soda to stand opened for several hours to allow the carbonation to escape

*Filter and vacuum aspirate to remove particles and trapped gas

* Standard Addition- Transfer volumetrically 1 mL of soda each to 5 vials, to the first add 1 mL of water, to each of the others add 1 mL of the prepared 1, 2, 3, and 4mg/mL caffeine solutions

*Calibration Curve- use straight soda

*Beer's Law- use straight soda; or dilute, as long as dilution is accounted for in subsequent dilutions

LOADING THE SYSTEM-

*Turn on the Waters 4000E using the On/Off switch on the lower right hand side of the instrument front. The ready light blinks indicating the machine is on and warming up. The light stops flashing after ten seconds.

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*Open the door by pushing the *Door Open* button above the On/Off switch. Note- do not stand directly over the unit, as the door opens upward.

*Place one vial of 0.1 M NaOH and one vial of water in the recessed vial holders located behind and to the left of the sample carousel to be used for purging and rinsing, respectively

*Remove the left hand side *sample carousel* as follows:

-Press the *Down Key* located at the center of the front panel of the unit, this lowers the carousel to its lowest point

-Take the *capillary block* off line by gently lifting it to its top most position, rotating it one quarter turn to the left and lowering it so the capillary end is suspended in one of the vials

-Unscrew the white *carousel knob* located in the center of the sample carousel

-Lift the *carousel cover* off

-Lift the carousel up and out of the unit

*Using a Pasteur pipette, fill the El *electrolyte reservoir* with the prepared buffer solution

*Fill the Limited Volume Inserts (LVI's) with the soda sample and the soda samples with addition of known caffeine solution:

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-The LVI's should be about 3/4 full, it is not necessary to measure the volume

-Tap or flick a finger against each LVI to dislodge any air bubbles

-Record the numbered location of each LVI as it is placed in the carousel

-Tuck the snap cap into the space provided on the carousel, so the cap will not interfere with the sampling process

*Replace the cover on the sample carousel and return it to the Waters 4000E, making sure to match the carousel bottom with the alignment pin, turn the carousel knob clockwise to secure the carousel

*Push the *Home* button, on the front panel to return the carousel to its home position

*Place a vial filled with buffer solution and sealed with a foil gasketed lid in the collection carousel in the #1 or Home position

PURGING

*With the capillary block rotated so the capillary end is in the *0.1 M NaOH* solution, push the manual purge button located on the front panel to the left of the number key pad. Allow the purge to run for approximately *three minutes.*

*Rotate the capillary block so the capillary end is in the *buffer solution*, push the manual purge button, and allow the this purge to run for approximately five *minutes.*

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*An auto purge may be programmed, but is probably not necessary with the small sampling numbers of this experiment.

*A purge should be performed between each repetition with buffer.

*The capillary should be rinsed with water at the end of the experiment to prevent crystal build up from salting out of the buffer.

SETTING PARAMETERS-

The program mode can be entered by pressing any of the function keys located on the front panel just below digital readout for each function. Enter a specific parameter as follows:

*Push *the function key*

*Enter the desired value using the *numerical keypad*

*Press enter

*Repeat for all parameter settings

*Press *Display Key* to exit program mode

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*Program the Following Parameters:

-Sample#: 1

-Repetition #: 2

-Sample Time: 10 sec.

-Run Time: 12 mm.

-Run Voltage: ~ 5.0 kV

-Sample Mode: Hydrostatic

-Purge: Manual

Note: All samples will run with the same parameters, so even though they are different they will be considered repetitions of the same sample (#1). Vary the number of repetitions to correspond with the number of different samples for each method (ex. 4 for a Calibration Curve, 5 for standard addition).

START THE RUN-

*Press the *Start/Stop* button

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*The ready light flashes for four seconds then the sampling begins

*Observe the Waters 4000E as the run progresses, monitor the voltage readout located at the top right of the digital display

*Observe the electropherogram, the caffeine peak should occur between 5.5 and 7 minutes after the start of each run

DISCUSSION:

The solution concentrations are based on an anticipated caffeine concentration in the soda of between 26 and 46 mg/ 355mL or 0.07 to 0.13 mg/mL (5,6).

The actual concentration of caffeine can be determined by plotting a graph of the concentration versus the peak height on the electropherogram for the Standards Addition and Calibration Curve methods, and extrapolating for the unknown concentration; or, through solving for the unknown concentration using the Beer's law formula A=CEL where E and L are constant and Absorbance is charted on the electropherogram.

PRE-LAB QUESTIONS:

1) What is the average content of caffeine in soda?

Ans: Sounds easy, but it actually took me several hours of tracking dead ends from Chemical Abstracts and Uncover listings. I finally found two sources recommended by a Northeastern librarian: the World Wide Web and the Nutrition Section (we just pulled a couple of books off the shelf and looked through the tables of contents. Anyway, the answer is 26-46 mg/355 mL

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2) How do you select the appropriate compound for a particular buffer solution?

Ans: Pick a form of acid based on the desired pH. For example, if you need a buffer with pH 7, look for an acid whose pK_a is close to 7. KH_2PO_4 has a $K_2=6.17 \times 10^{-8}$, with a pH of 7.21 is a good choice.

3) Calculate the number of grams of salt needed to prepare the required buffer solution.

Ans: g. salt = (# liters)(formula weight of salt, g/mol)(Molarity)

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