Capillary Electrophoresis of Water-Soluble Vitamins: An Undergraduate Experiment

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Abstract: Capillary electrophoresis (CE) is a relatively new analytical separation technique that is not usually introduced in the undergraduate analytical chemistry curriculum. The technique's growing popularity in research, industrial, and commercial laboratories, however, should be a reason to consider its introduction at this level. Here, we describe an exercise utilizing capillary zone electrophoresis and micellar electrokinetic chromatography. This exercise provides a suitable introduction to capillary electrophoresis and illustrates the mechanism for the separation of ionized and nonionized water-soluble vitamins (B1, B2 phosphate, B3 niacinamide, and B12). Joule heating can also be easily introduced as part of the exercise.

Introduction

Separation techniques are of considerable chemical interest, and it is necessary to introduce chemistry students to these techniques. As well as the chromatographic methods (liquid chromatography and gas chromatography), which are traditionally the separation techniques taught at the undergraduate level, it would be helpful to teach capillary electrophoresis (CE) because of the large number of applications for this powerful separation technique in industry and research. Several recent reports have proposed useful CE experiments that may be incorporated into the analytical chemistry curriculum. [1–13]

Unlike other separation techniques, the simplicity of the underlying principles of CE makes it easy for students to understand. An advantage of CE as a teaching tool is that it provides a high degree of separation with a low consumption of reagents and solvents. The high efficiency of CE is a result of electro-osmotic flow (EOF), the movement of the electrolyte solution towards the cathode when a voltage is applied. The velocity of the EOF depends on the pH of the buffer electrolyte, the higher the pH the higher the velocity. Separations by CE are based on differences in electrophoretic mobility of the solutes when a strong electric field is applied across a separation buffer solution. The mobility of the solute (μ_{ep}) in the separation medium depends on its charge and ionic radius:

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

where q is the solute charge, r is the ionic radius, and η is the viscosity of the electrolyte . The combination of EOF and the electrophoretic mobility of the solute results in the apparent mobility of the solute (μ_{app}) towards the cathode:

$$\mu_{app} = \mu_{eof} + \mu_{ep}$$

The prediction of the separation behavior in CE, in which nothing is retained, is easier than for chromatographic separation techniques, where solutes are retained in the column. In CE, it is only necessary to know the charge/size ratio of each solute.

In addition to the high efficiency of CE, it is also considerably faster than other chromatographic techniques, and, therefore, several analyses may be performed in a single laboratory session. CE capillaries are simpler to wash and condition than the chromatographic columns used in GC and LC.

The usefulness of the simplest CE mode, capillary zone electrophoresis (CZE), is limited to the separation of ionized compounds in solution; however, it is possible to obtain effective separations of nonionized water-soluble compounds through the addition of a surfactant, usually anionic sodium dodecyl sulfate (SDS), to the buffer solution. This CE mode, which is known as micellar electrokinetic chromatography (MEKC), is in effect a combination of electrophoresis and chromatography. Separation by MEKC is dependent on the existence of a pseudostationary phase, which is formed by the micelles created in the separation electrolyte when the surfactant is added at a concentration higher than its critical micelle concentration (Figure 1). The order of separation of neutral compounds is determined, from low to high, by their hydrophobicity within a finite window.

A concept that is necessary to explain to students in a CE laboratory session is Joule heating. This takes place when the system is unable to dissipate the heat generated in the capillary. The quantity of nondissipated heat is not consistent from one run to another, and this fact results in a lack of reproducibility in the migration times of the solutes. Students may find the theoretical explanations of Joule heating to be difficult to understand and, unfortunately, none of the reports that introduce CE to the analytical curriculum introduce this effect [1–13]; however, the fast and simple experiment that we present here can be used to illustrate clearly to students the formation of Joule heating and its effect on migration times.

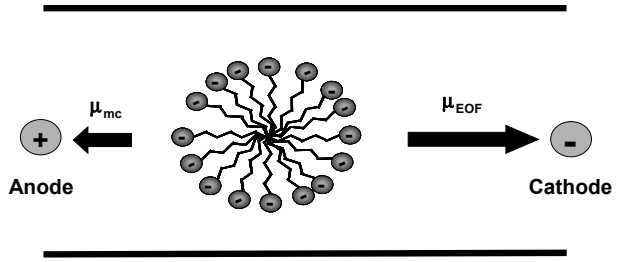


Figure 1. Scheme of a SDS micelle and its movement in a capillary. μ_{eof} is the electroosmotic mobility and μ_{mc} is the micelle mobility. Basic media is needed to allow micelles to move to the cathodic end of the capillary ($\mu_{eof} > \mu_{mic}$).

Several texts are available for those who require more detailed theory than that given here [14–16] and an excellent introduction to CE can be obtained through some reviews [17–21] and a web page [22].

The aim of this paper is to present a fast and inexpensive one-day laboratory that introduces students to the basics of capillary electrophoresis as well as the use of the MEKC mode to separate neutral solutes. Furthermore, Joule heating is introduced.

Experimental

Preliminary Remarks. This experimental protocol has been developed with the understanding that the students will have had one year of general chemistry laboratory. It will be necessary to present a prelaboratory lecture on capillary electrophoresis with an introduction to MEKC.

Safety. Safety goggles must be worn if the capillary column is removed from the instrument to show to students. Students should be advised to dry the external surfaces of vials before placing them in the carrousels in order to avoid electrical discharges when high voltages are applied.

Electrophoretic Solutions. For capillary zone electrophoresis experiments, a 40 mM sodium borate/boric acid buffer at pH 8.5 is used. At this pH, the electro-osmotic flow is directed towards the cathodic end of the capillary and the detection of anions is made possible as the velocity of the flow exceeds their migration velocity.

For the MEKC experiments, it is necessary to prepare two buffer solutions of 40 mM sodium borate/boric acid at pH 8.5, one with 20 mM of SDS as a surfactant and the other with 40 mM of SDS. The alkaline pH is necessary so that the electro-osmotic flow will be faster than the micellar velocity and hence allow the detection of solutes with a high affinity to the micelles.

Samples. Four water-soluble vitamins with different ionized forms at pH 8.5 are selected for the experiments (B1, B2 phosphate, B3 niacinamide, and B12) (Figure 2). Vitamin B1 is positively charged at pH 8.5, vitamins B3 and B12 are neutral, and vitamin B2 phosphate is negatively charged. Different vitamin-containing samples (e.g., juices, multivitamin preparations) may be supplied to students.

Instrument and Conditions

Experiments were carried out with a Waters Capillary Ion Analyzer (CIA) with an unmodified fused-silica capillary of 63 cm (57 cm to the detector) with a 75-μm internal diameter. All the electrophoretic measurements were made at 25 °C and detection was made at 254 nm. Twenty-second hydrodynamic injections are required.

In order to determine the migration time of each vitamin, runs of each individual vitamin must be made in each mode of analysis. The migration times obtained allow students to assign the peaks to the corresponding individual vitamins when the vitamin mixtures are analyzed. The number of runs can be reduced by the use of a CE instrument with a photodiode-array detector, which makes it possible to directly associate peaks by the difference in the UV spectra of the vitamins evaluated.

Results and Discussion

Capillary Zone Electrophoresis Experiments. CZE is based on the movement of charged molecules within the capillary when an electric field is applied. Solute electrophoretic velocity (v_{ep}) is governed by the following equation

$$v_{ep} = \mu_{ep} \quad E = \mu_{ep} \frac{V}{L_T}$$

where μ_{ep} is the electrophoretic mobility of the solute that is the determining factor (eq 1), E is the applied electric field, V is the applied voltage, and L_T is the total capillary length.

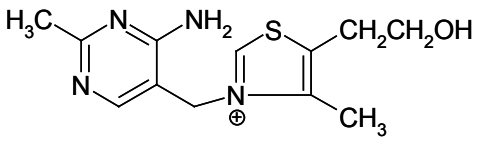
Students first evaluate the effect of the applied voltage over the electrophoretic velocity and mobility of the solutes. Prior to the first run, students are asked to predict the number of peaks and the separation order of the vitamins in the sample at each voltage applied. Then, a sample containing a mixture of all the vitamins is separated at four different voltages, ranging between 10 and 25 kV, using the 40 mM borate buffer as electrolyte solution. It should, in fact, be easy for students to determine that only three peaks will appear in all the electropherograms as there are two neutral vitamins in the mixture that cannot be separated by CZE. The only way solute electrophoretic mobility can be calculated is experimentally:

$$\mu_{ep} = \left(\frac{1}{t_T} - \frac{1}{t_0} \right) \cdot \left(L_D \frac{L_T}{V} \right)$$

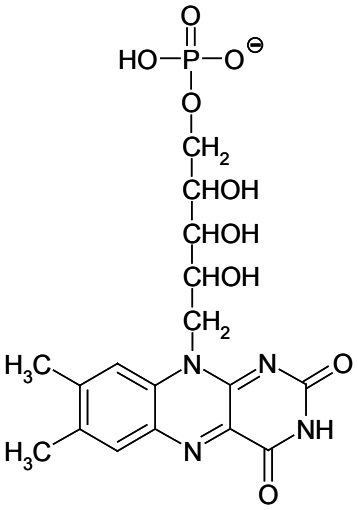
where L_D is the distance between the point of injection and the point of detection, L_T is the total length of the capillary, V is the separation voltage, t_0 is the migration time of the electroosmotic flow (determined from the migration time of a neutral solute), and t_T is the migration time of the solute.

The same elution order is obtained at all voltages as the electrophoretic mobility is not voltage dependent. According to eq 1, electrophoretic mobility only depends on the charge/size ratio of solutes, but it is possible to correlate electrophoretic mobility directly with charge for mixtures containing solutes with different charges, as in the case study. Hence, the first peak, which corresponds to the faster solute, is associated with vitamin B1 due to its positive charge ($\mu > 0$). The second peak corresponds to an overlap of the peaks of neutral vitamins B3 and B12 ($\mu = 0$). The final peak is due to vitamin B2, which is negatively charged ($\mu < 0$).

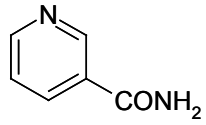
Next, students are told to determine the best voltage to separate a mixture of only three vitamins, now with only one of the neutral vitamins. They normally choose 25 kV because at this voltage the three peaks are baseline-separated and the total analysis time is shorter (4.5 min at 25 kV as compared to 10 min at 10 kV). This choice leads students to an investigation of Joule heating, which affects the



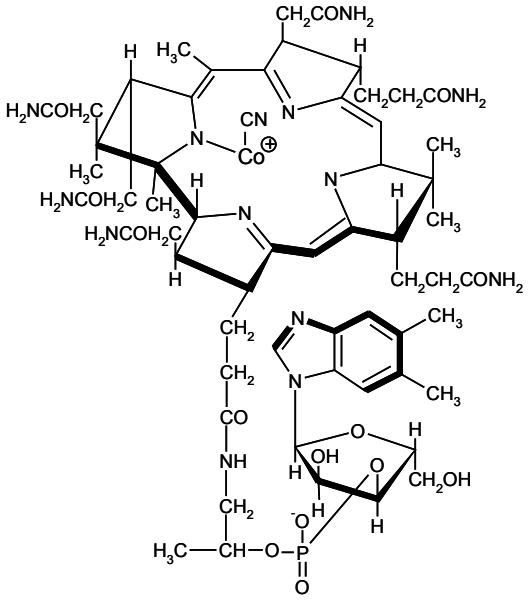
Vitamin B1



Vitamin B2 phosphate



Vitamin B3 (niacinamide)



Vitamin B12

Figure 2. Structure of the four vitamins evaluated.

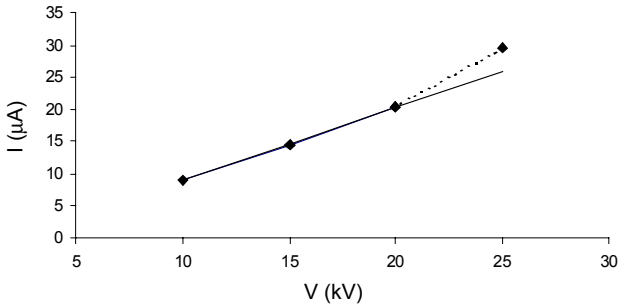


Figure 3. Ohm's law graph permits the determination of the formation of Joule heating when a deviation from linearity is observed.

reproducibility of the migration times at the highest voltage. Students need to note the current generated in the system during each run as the different voltages are applied. As resistance (R) is constant in all runs, Ohm's law ($V = IR$) tells us that a linear graph should be obtained when the applied voltage is plotted against the current. Due to the Joule heating effect, however, when students plot their data (Figure 3) there is a deviation from linearity at higher voltages. It will be seen from the resulting graphs that 20 kV is the maximum voltage that can be applied to obtain reproducible migration times.

MEKC Experiments. In order to obtain the separation of the two neutral vitamins in the mixture, a different mode of CE must be selected. In the MEKC mode a surfactant is added to the background electrolyte solution, which allows separation of both charged and neutral solutes in a single run. In this case, SDS (sodium dodecyl sulphate) is added as the surfactant to the original sodium borate/boric acid buffer solution. A 4 to 5 min condition time is required before separation can be attempted.

SDS micelles have a very slow migration velocity due to their negative charge (Figure 1). The migration velocity of solutes in MEKC depends on their degree of interaction with the micelles. The greater the interaction, the slower the migration velocity (longer migration time). All solutes appear in the so-called "migration window," which is the period between the migration time of the electro-osmotic flow and the migration time of the micelle itself. In the case of MEKC, the partition coefficients that are involved make it more difficult for students to predict the separation order than in CZE. Still, the hydrophobicities of the neutral vitamins used in this study are sufficiently different to enable students to make a theoretical prediction. These MEKC experiments are designed both to evaluate the separation efficiency of this mode and to study the effect of the surfactant concentration on peak resolution and analysis time.

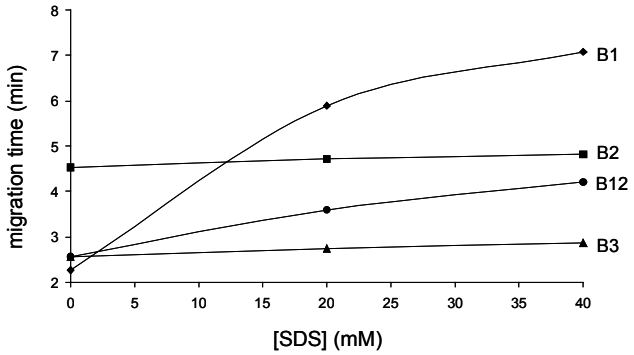


Figure 4. Evolution of the migration time of the four water-soluble vitamins at different concentrations of surfactant in the background electrolyte (Voltage applied = 20 kV).

Students are asked to predict the separation order of the vitamins in MEKC mode under the new conditions before analysis. The main problem is the position of the negatively charged vitamin in the series. It is relatively easy for students to understand that the neutral and positively charged vitamins have to appear in the electropherogram in the order: vitamin B3, B12, and B1. Vitamin B1 appears last because of the formation of ion pairs between the negatively charged SDS micelles and the positively charged vitamin [23]. The two neutral vitamins, B3 and B12, are separated according to their hydrophobicity. The most hydrophilic vitamin, B3, appears faster because it has a lower affinity to the micelles. Vitamin B12 is more hydrophobic and has a higher partition coefficient in the micelles, leading to a greater migration time in the capillary. The anionic vitamin B2 phosphate has little affinity for the micelles and it retains similar mobility without the use of a surfactant. A slight change in the migration time is expected with respect to the migration time obtained in the CZE measurements because of the change in the viscosity that results from the addition of the surfactant. The overall determination of the exact peak position is complicated by the addition of the surfactant and the different affinities of the solutes towards the micelles.

The use of 20 mM SDS BGE allows the separation of the two neutral vitamins and hence a four-peak electropherogram is obtained when the vitamin mixture is analyzed. Students are also required to analyze a vitamin sample using a higher concentration of SDS in the electrolyte (40 mM), which increases the quantity of micelles in the electrolyte. This increases the solute–micelle interactions and results in increased migration times for each solute (Figure 4). It will be observed that the variation in migration time for the cationic vitamin B1 is highest, followed by B12, the most hydrophobic neutral vitamin. The migration time of vitamin B3 varies little, as its partition coefficient in the micelles is smaller. Vitamin B2 has the same migration time at the two different concentrations of surfactant because it has little interaction with the micelles and its mobility is not affected by the addition of a negative surfactant.

Conclusions

This proposed laboratory procedure introduces students to the main concepts associated with capillary electrophoresis separation techniques. The effectiveness of this activity is increased by exercises requiring students to predict the outcome and by short analysis times. The data that students obtain help them to understand the theoretical knowledge being taught.

To facilitate the speed of analysis it is suggested that all reagents and samples be prepared for the students. Further investigations could include the analysis of other vitamin-containing solutions such as vitamin C, which is a common and easily identifiable solute. We encourage students to try to hypothesize the separation order in all samples before separation. It is also suggested that data analysis obtained by different groups be discussed by the entire group to permit better understanding of this technique and further evaluation of good laboratory practices.

Supporting Materials: A student handout for the Capillary Electrophoresis experiment is available as Supporting Material s00897020532b.pdf.

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