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Role of buffer concentration and applied voltage in obtaining a good separation in planar electrochromatography

David Nurok*, James M. Koers, Matthew A. Carmichael
Department of Chemistry, Indiana University Purdue University Indianapolis, 402 N. Blackford Street, Indianapolis, IN 46202, USA

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Abstract

Planar electrochromatography is performed by applying an electric field across a thin layer chromatography (TLC) plate. In addition to electroosmotic flow in the axial direction, there is also flow to the surface of the TLC layer, and this can substantially degrade the quality of separation. This effect is offset by Joule heating which causes evaporation of liquid from the layer surface, and which under some conditions causes degradation of separation quality by excessive drying of the layer. It is shown that pH, buffer concentration, and applied voltage control the balance between liquid being driven to the surface and liquid evaporating from the surface due to Joule heating. Conditions are discussed which result in good separation quality, or in separations degraded by either excessive wetting or drying of the layer. The above separations were performed at constant voltage. A chromatogram is presented that shows that a good separation is also obtained at constant power, i.e. under conditions where there is a constant amount of Joule heating.

Keywords: Electrochromatography; Planar electrochromatography; Buffer composition; Benzoic acids; Nitroaniline; Benzanilide; Acetonaphthone; Acetoxyprogesterone; Cholestenone

1. Introduction

Planar electrochromatography (PEC) is a technique in which electroosmotic flow is used to drive the mobile phase in thin layer chromatography (TLC). The first description is by Pretorius et al. [1] in 1974, who referred to it as high speed thin layer chromatography. After a long hiatus several reports have been published using either pre-wetted [2–6] or initially dry [7–11] layers. The current report refers to a pre-wetted layer, and the use of an initially dry layer is not discussed further.

Under appropriate conditions, separation by PEC is substantially faster than an equivalent separation by classical TLC. The report by Pretorius et al. described a separation that required 4 min by PEC and 60 min by classical TLC. Howard et al. [6] have reported a similar enhancement in speed of analysis. Other reports describe separations where the diminution of separation time is less substantial [2–4].

The technique should have several advantages over classical TLC, in addition to the speed of an analysis. The flow rate should ideally be dependent on variables such as buffer salt concentration and the

*Corresponding author. Tel.: +1-317-274-6886; fax: +1-317-274-4701.
E-mail address: nurok@chem.iupui.edu (D. Nurok).

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applied electric field, and should be independent of the mobile phase path length. In practice these advantages have not been realized. Nevertheless the technique has potential to perform useful separations, as illustrated by a recent report of a virtually baseline separation of six compounds in 10 min [5], with good reproducibility.

PEC is very sensitive to operating conditions, and in the report below we discuss the importance of using the correct combination of buffer concentration, pH, and applied voltage.

2. Experimental

Merck RP-18 F_{254}s plates (EM Science, Gibbstown, NJ, USA) were cut to 2.0 cm×10 cm and conditioned in an oven at 120 °C for 20 min. The plates were stored in a desiccated box and used within 24 h of conditioning. Solutes were dissolved in acetone, and were spotted on the layer using a Camag Nanomat II fitted with a Camag Nano Applicator (Wilmington, NC, USA) set to deliver 75 nl.

Acetonitrile, 2-amino-2-hydroxymethyl-1,3-propanediol (Tris) base, Tris–HCl, acetic acid and sodium acetate were purchased from Fisher Scientific (Pittsburgh, PA, USA), and benzanilide, 17-α-acetoxyprogesterone, 2′-acetonapthone, *p*-hydroxybenzoic acid, 3,4-dimethoxybenzoic acid, and *o*-nitroaniline were a gift of Don Risley of Eli Lilly and Company. The 4-cholesten-3-one was from Mann Research Labs. (New York, NY, USA). The water was filtered with a Milli-Q system.

Both the acetate and the Tris buffer solutions were prepared at the desired pH by mixing a 1 *M* solution of the basic form and a 1 *M* solution of the acidic form of the appropriate compound. This procedure was monitored using a pH meter. The concentration of the buffer solution was then adjusted such that, when mixed (on a volume/volume basis) with acetonitrile and additional water, the mobile phase was at the desired molarity. The pH values reported are for the solutions obtained by mixing the respective 1 *M* stock solutions prior to addition of acetonitrile and additional water, and for this reason these are referred to as nominal pH values.

A slightly modified DS-II horizontal chamber (Chromdes, Lublin, Poland), described in an earlier report [5], was used for performing PEC. Ref. [12] describes the unmodified Chromdes chamber.

The TLC layer is positioned facedown in the apparatus, with a saturation trough beneath it. This was filled with mobile phase to allow vapor saturation and prevent excess evaporation from the layer surface.

Prior to each run the horizontal chamber was leveled using an air bubble in oil device. The eluent chambers and the saturation chamber (beneath the TLC plate) were then filled with the run buffer solution. Each plate was pre-wetted before PEC using a Desaga (Heidelberg, Germany) dipping chamber that contained the same mobile phase as used for the separation. The plate was wetted to within approximately 1 mm of the initial spot position. Excess mobile phase was removed from the silica layer by blotting on a paper towel, before placing the TLC plate in the developing chamber. The cover plate and ‘‘eluent distributors’’ were then placed in position and the ‘‘eluent distributors’’ moved to initiate contact between the pools of mobile phase and the TLC layer. The power source [Bio-Rad Power/Pac 3000 d.c. supply (Hercules, CA, USA)] was switched on as soon as the mobile phase reached the analyte spots.

The plates were allowed to dry after PEC, and were then scanned at $\lambda = 254$ nm with a Shimadzu (Kyoto, Japan) CS9000U dual wavelength flying-spot scanner in the reflectance mode.

3. Predictive equations

Electroosmotic flow in a channel is predicted by the following equation [13]:

$$u_{eo} = \frac{\varepsilon_r \varepsilon_o \zeta E}{\eta} \quad (1)$$

where ε_o is the permittivity of vacuum, ε_r is the dielectric constant, ζ is the zeta potential, E is the electric field and η is the viscosity of the mobile phase. This equation assumes that the size of the channel, in which flow occurs, is large as compared to the size of the electrical double layer.

The value of ζ is given by [14]:

$$\zeta = \sigma \sqrt{\frac{RT}{2\varepsilon_r \varepsilon_o c F^2}} \quad (2)$$

where σ is the charge density at the surface of sheer, R is the gas constant, T is the absolute temperature, c is the molar concentration of the buffer salt, and F is the Faraday constant.

These equations predict that electroosmotic flow will decrease with increasing electrolyte concentration, and this is indeed found to be correct in capillary electrophoresis [15].

In PEC, however, electroosmotic flow increases with increasing buffer concentration [4], which is contrary to what is predicted by the above equations. Reports on capillary column electrochromatography (CEC) indicate that there is a diminution of electroosmotic flow with increasing buffer concentration for all, or part of the concentration ranges studied [16–20]. A report by Banholczer and Pyell [16] describes conditions where electroosmotic flow initially increases, and then decreases, with increasing buffer concentration.

The size of the electrical double layer, δ, is inversely related to the concentration of the buffer by the following relationship [21]:

$$\delta = \sqrt{\frac{\varepsilon_r \varepsilon_o RT}{2cF^2}} \quad (3)$$

Wan has explained the diminution of electroosmotic flow with decreasing buffer concentration in terms of an overlap of the electrical double layer in narrow channels when using a low buffer concentration [20]. This author has also published a theoretical analysis [22] comparing electroosmotic flow, with and without, double layer overlap. This analysis showed that this effect can be substantial for a column packed with 5 μm particles at low buffer concentration. These results are not in agreement with Knox and Grant who found that electroosmotic velocity is virtually independent of the particle diameter, and that double layer overlap is not an important parameter [18]. In the discussion below we use Wan's interpretation of double layer overlap.

4. Results and discussion

There are a number of important experimental parameters that influence the quality of a separation in PEC. In a vertical chamber, as used in Refs. [2–4], the placement and size of the wick is important. Frost [23] noted that if the wick is not correctly placed, or is of insufficient size, the flow of the mobile phase is impeded in the forward direction, and electroosmotic flow drives liquid to the surface of the TLC layer. This results in the spots streaking badly. This streaking can also occur in an apparatus without a wick, as described in the current report for separations in the horizontal DS chamber.

The wetting of the layer is observed under only certain conditions [23] when using the regular Merck RP-18 plates with either aqueous acetonitrile or aqueous ethanol, containing a suitable buffer, as the mobile phase. The wetting occurs over a substantially wider range of conditions with the Merck RP-18W plates. The ''W'' sorbent has a higher concentration of residual silanol groups, and this results in a larger electroosmotic flow for any given set of experimental conditions.

The same problem occurred when PEC was performed in the normal-phase mode [24] on a silica gel layer using acetonitrile–toluene (20:80, v/v) as mobile phase with 25 m*M* tetrabutylammonium bromide as an additive. The same vertical chamber was used as for the above experiments. Electroosmotic flow in the normal-phase mode requires a higher applied voltage than in the reversed-phase mode. Substantial streaking was observed at applied voltages above 8 kV. Electroosmotic flow was very slow at lower voltages, and for these reasons the approach was not further explored.

In addition to separation by electroosmotic flow, separation also occurs by other mechanisms in the modified DS chamber. Two of these are due to capillary mediated flow as a result of the sorbent layer not being fully saturated with mobile phase. Shafik et al. [25] have proposed a mechanism where this unsaturation is due to evaporation of the mobile phase as a result of Joule heating. (An additional effect of evaporation is the generation of a solvent gradient, which has the effect of sharpening the peaks [5].) A second cause of unsaturation is due to the blotting of the layer before the TLC plate is placed in the apparatus. In the case of charged species, electrophoretic effects must also be considered. As an example, the two compounds of highest

R_F in the test mixture are acids, and will be present as anions at high pH. The electrophoretic contribution to migration for these two negatively charged species will be towards the anode, i.e. in the opposite direction to electroosmotic flow. Even though these are the two species of highest R_F in the mixture, their migration relative to the other analytes is lower in PEC than in TLC with the identical mobile phase. While there are other possible interpretations of this result it strongly suggests the presence of an electrophoretic effect. The individual contributions of the above effects to migration are most probably small as compared to electroosmotic flow, and are ignored in order to simplify the discussion below.

The horizontal DS chamber has a reservoir of mobile phase at each end of the TLC plate, with the liquid from the reservoirs connected to the layer surface via two capillary channels. Under certain conditions liquid is observed on the layer surface, and this cannot be related to any shortcomings in the wicking arrangement, as there is no wick.

The following explanation is offered to explain why liquid is driven to the surface. In any packed bed there is a distribution in the size of the channels through which the liquid flows, and under certain conditions the flux of liquid from one channel to the next may be substantially different. In a packed tube the channels of lower flux will influence the overall electroosmotic flow. In an open system, such as in PEC, there is an additional effect. When a channel of higher flux leads to a channel of lower flux the excess liquid can migrate towards the surface, as there is no constraining pressure. Liquid will accumulate on the surface if this effect is sufficiently large. The differences in flux may be due to different degrees of electrical double layer overlap in different channels, or simply due to differences in size of the channels.

In order to investigate the conditions under which wetting of the surface occurs, a test mixture was separated on each of seven Merck RP-18 plates using 55% aqueous acetonitrile as the mobile phase. The compounds in the mixture are, in the order of increasing R_F, 4-cholesten-3-one, 17-α-acetoxyprogesterone, 2′-acetonapthone, benzanilide, *o*-nitroaniline, 3,4-dimethoxybenzoic acid, and *p*-hydroxybenzoic acid. A different concentration of acetate buffer at a nominal pH of 4.5 was used for each separation, which was performed for 10 min, with the exception of those at the two highest acetate concentrations. These separations are shown in Fig. 1.

In the context of this discussion, a good separation is defined as one in which all spots were separated and there was no wetting of the surface or drying of the layer. Wetting was evidenced either by substantial streaking (see Fig. 1, 5.0 m*M* buffer concentration) or blurring of spot shape, and the drying was evidenced by a substantial drop in current followed by visible drying of the layer. At the two lowest acetate concentrations (1 m*M*, 5 m*M*) there is clear evidence of streaking due to accumulation of some liquid on the layer surface. Good separations are obtained at the intermediate acetate concentrations (10 m*M* to 25 m*M*). At a 50 m*M* and 100 m*M* acetate concentration the plate dries at approximately 4 and 2 min, respectively. The interpretation of this behavior is as follows. Joule heating increases with increasing electrolyte concentration in the mobile phase (i.e. with increasing buffer concentration), while the overlap of the electrical double layer will diminish with increasing electrolyte concentration.

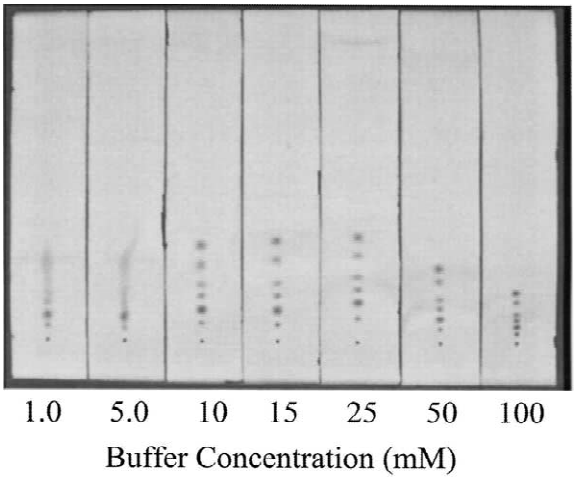

Fig. 1. Separation of a seven component mixture on a RP-18 layer at 1000 V using, as mobile phase, 55% aqueous acetonitrile containing acetate buffer at a pH of 4.5. The buffer concentrations are as indicated. In order of increasing R_F, the compounds are: 4-cholesten-3-one, 17-α-acetoxyprogesterone, 2′-acetonapthone, benzanilide, *o*-nitroaniline, 3,4-dimethoxybenzoic acid, *p*-hydroxybenzoic acid.

This diminution in overlap should diminish the tendency of mobile phase to migrate to the surface.

The overall result is that low Joule heating results in inadequate evaporation and accumulation of liquid on the surface of the layer at low buffer concentration. At intermediate buffer concentration there is a balance between evaporation from the surface and the migration of liquid to the surface. This results in optimum conditions for PEC. At high buffer concentration the heating and resulting evaporation predominates, and the layer dries prematurely.

A similar effect is achieved when working at a constant buffer concentration over a range of pH values. Thus when the 5 m*M* separation was repeated at a pH of 8.4 instead of a pH of 4.5 there was no evidence of streaking and the separation was very similar to that obtained in the 10 m*M* to 25 m*M* range in Fig. 1. At a pH of 4.5 the acetic acid is about 50% ionized (assuming that the behavior is similar in a completely aqueous solution and in the mobile phase), whereas at a pH of 8.4 it is fully ionized. Thus more Joule heating and more resultant evaporation occurs at the higher pH. Using a similar argument when the pH of the 10 m*M* separation is changed from 4.5 to 2.4 the good separation shown in Fig. 1 degrades and a poorer separation with evidence of streaking occurs. At low pH there is very little ionization of the acetic acid as compared to that at a pH of 4.5, and thus little Joule heating occurs, and evaporation is diminished. Migration distances of the spots are lower at a pH of 2.4 due to lower electroosmotic flow, which in turn is due to both a lower concentration of electrolyte and a lower ionization of silanol groups. It is expected that the lower electroosmotic flow lessens the flux of liquid to the layer surface, but this effect appears secondary to the substantially reduced Joule heating. In other words, the most important effect of lowering the pH appears to be the lowering of evaporation rather than the lowering of the amount of liquid driven to the surface.

Streaking does not always occur at low pH. Separations with 55% aqueous acetonitrile containing 25 m*M* acetate buffer did not exhibit any evidence of streaking at the following pH values: 2.4, 3.4, 4.5, and 8.4. The separations at the two lower pH values were virtually identical, as were the two separations at the higher values, but the distances migrated by the spots were substantially higher at the two higher pH values, and this resulted in better overall resolution at these pH values. The separation at pH 4.5 is shown in Fig. 2a.

A larger set of experiments was performed using Tris buffer and a mobile phase consisting of 65% aqueous acetonitrile. The results with Tris differ from those with acetate buffer, because the parent compound is a base and has a positive charge at low pH and no charge at high pH. Separation at a 30 m*M* Tris concentration and a pH of 7.8 is shown in Fig. 2b.

Fig. 3 shows two plots of separation quality as a function of applied voltage and nominal pH, using 65% aqueous acetonitrile containing Tris buffer at two different concentrations. The letters G (good), W (wet), and D (dry) are used to describe separation quality. The separation varies from ''dry'' to ''good'' to ''wet'' as the pH is increased from 4.0 to 10.5 at 800 V for the 30 m*M* concentration, and at 1500 V for the 15 m*M* concentration, respectively. At a nominal pH of 4.0 Tris should be predominantly in

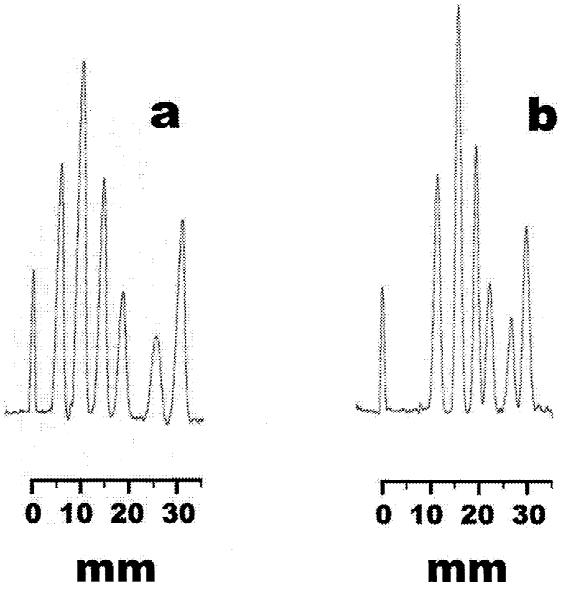

Fig. 2. (a) Separation of the same solute mixture as in Fig. 1 on a RP-18 layer at 1000 V using, as mobile phase, 55% aqueous acetonitrile containing 25 m*M* acetate buffer at a pH of 4.5, (b) Separation of the same mixture on a RP-18 layer at 800 V using, as mobile phase, 65% aqueous acetonitrile containing 30 m*M* Tris buffer at a pH of 7.8.

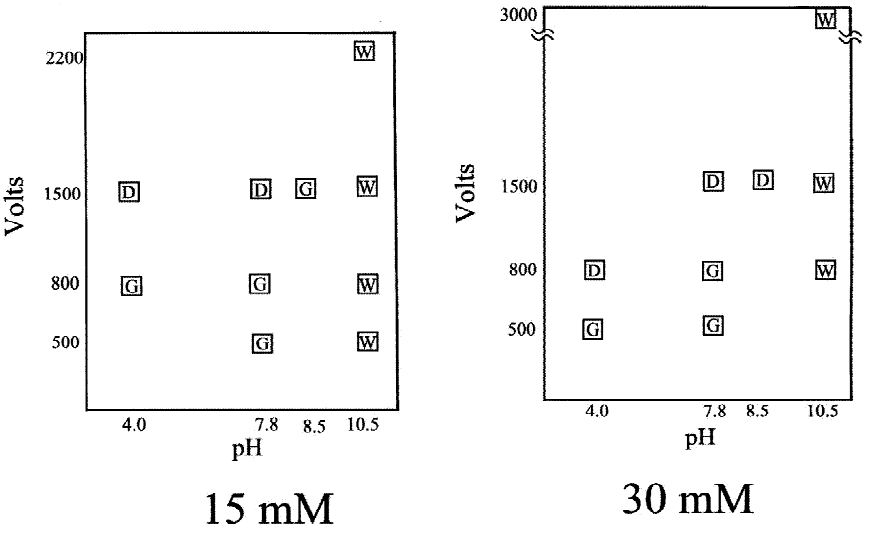

Fig. 3. Plots indicating the separation quality obtained for the seven compounds (see Fig. 1) at different combinations of applied voltage and pH using either 30 m*M* or 15 m*M* Tris buffer. ''D'' indicates that layer drying was observed, ''G'' indicates that a good separation, without any evidence of either streaking or drying, was observed, and ''W'' indicates that layer wetting and spot streaking were observed.

the ionic form. This leads to substantial Joule heating at both of the above voltages, and this causes drying of the layer. Lowering the voltage at this pH, and at either buffer concentration, reduces the Joule heating and results in a good separation. Instead of lowering the voltage a good separation can be obtained by raising the pH to 7.8 (for 30 m*M*, 800 V) or 8.5 (for 15 m*M*, 1500 V). The raise in nominal pH to the vicinity of the pK_a of the Tris (7.8) reduces the degree of ionization of the buffer. This results in lower Joule heating, and also in an increase in the dimensions of the double layer. The latter may result in more liquid being driven to the surface. The net result is that the layer does not dry under these conditions.

Increasing the pH to 10.5 results in wetting of the layer for all combinations of buffer concentration and voltage. At this pH, the amount of liquid driven to the surface is greater than the amount removed by evaporation at all applied voltages studied. The diminution of Joule heating is evidenced by a decrease in current with increasing pH at all voltages for either of the buffer concentrations considered.

4.1. Operation at constant power

Due to evaporation, the composition of the mobile phase will not be uniform across the length of the TLC plate, and thus the electrical resistance and the electric field (i.e. volts/cm) will vary across the length of the plate. For this reason the above results are discussed in terms of voltage, even though electroosmotic flow is governed by the electric field (see Eq. (1)). During a PEC run at constant voltage the electrical current is not stable, and the resultant power and Joule heating fluctuates. In order to stabilize the system, an attempt was made to perform PEC at constant power. Under these conditions both the current and voltage can fluctuate, but their product (i.e. the power in watts) remains constant, as does the amount of Joule heating. Preliminary results indicate that a good separation is obtainable, as illustrated in Fig. 4, which shows a PEC separation at 3 W using 55% aqueous acetonitrile containing 100 m*M* acetate buffer at a pH of 4.5. In replicate runs the TLC plate dries in the range of 3.5 min to 4 min, and different experimental conditions will be required to obtain better reproducibility using this approach.

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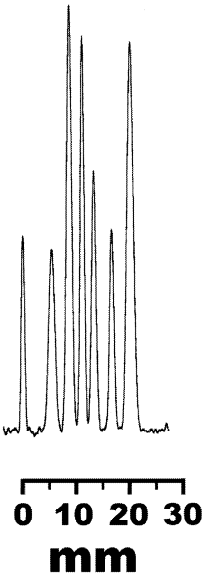

Fig. 4. Separation of the same solute mixture as in Fig. 1 on a RP-18 layer at 3.0 W using, as mobile phase, 55% aqueous acetonitrile containing 100 m*M* acetate buffer at a pH of 4.5.

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