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# Field programming to achieve uniform sensitivity for on-line detection in electrophoresis

Under constant applied electric field, an analyte band traverses an on-line detection zone in an electrophoretic separation at a velocity inversely proportional to the detection time. Because analyte bands migrate with different velocities, the sensitivity of the detection system to different species can vary significantly. One approach for overcoming this problem is to change the applied electric field, $E$, as a function of the separation time, $\tau$, so that the velocity of an analyte band when it passes through the detection zone is independent of the analyte's electrophoretic mobility. A possible solution is to make $E(\tau)$ grow exponentially, but this procedure causes band compression. The preferred solution is to make both $E(\tau)$ and the distance between the starting position of the sample and the detection zone increase linearly with separation time. This approach can be implemented with either slab-gel or capillary electrophoresis.

For electrophoretic separations performed using a constant electric field, the velocity at which a band passes through an on-line detection zone, $v_{det}$, is inversely proportional to the elapsed time between the start of the separation and detection, $\tau_{det}$:

$$v_{det} \propto 1/\tau_{det} \qquad (1)$$

Accordingly, the time available for measurement as analyte molecules pass through the detection zone is not constant, and as a consequence, detection sensitivity for on-line measurements in electrophoresis can change significantly throughout the course of a separation. When employing fluorescence detection* that is limited by shot-noise in the background, maximum sensitivity for electrophoresis is achieved when the excitation intensity results in approximately 70% photoalteration of the analyte and when data points are generated by binning the detector signal for the longest acceptable period [1]. Because the residence time of analytes in the detection zone determines the excitation intensity and data digitization rate that must be employed to achieve maximum sensitivity, these factors will not be optimized at all separation times if they are held constant throughout a separation. Previously we have demonstrated that if the excitation intensity and the data digitization rate are optimized for the fastest band in a separation, uniform sensitivity with the best possible signal-to-noise ratio (SNR) is achieved when the excitation intensity (or the probe volume) and data digitization rate are decreased after the fastest band is detected by the factor $\tau_{fast}/\tau$. Here, $\tau_{fast}$ is the arrival time of the fastest band and $\tau$ is the elapsed time since the separation was started [1].

These manipulations of excitation intensity and data digitization rate would be unnecessary if all analyte bands migrated through the detection zone at the same velocity. This paper explores possible strategies for achieving this goal. One approach is to program the separation field, $E(\tau)$, to evolve during the separation so that the mobility of an analyte species is inversely related to the magnitude of the field at the time the analyte reaches the detection zone. We demonstrate that an approximate solution is obtained when $E(\tau)$ increases exponentially with $\tau$. This solution, however, results in significant band compression, and the effects on analyte resolution can be prohibitive. A superior approach that results in a uniform detection velocity with no band compression is to vary linearly with $\tau$ both $E(\tau)$ and the distance between the starting position of the sample and the detection zone.

In this work, the assumption is made that the electrophoretic migration velocity and the electroosmotic flow velocity, if it exists, are related linearly to the separation field. Although this is an idealization [4], it is fairly accurate for small or moderate-sized molecules as long as the mobilities of analytes and the properties of the electrophoresis medium do not undergo significant temperature-dependent changes within the range of separation field strengths employed.

When employing a stationary detection zone, there exists no closed-form function for ramping the field that can generate an absolutely uniform detection velocity for all analytes.** Nevertheless, a nearly uniform detection

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**Abbreviations: SNR,** signal-to-noise ratio

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\* The same considerations are applicable to absorption detection; however, lamp sources are often incapable of generating the irradiance necessary to cause significant photoalteration of analyte molecules, and hence, slower bands can be detected with better sensitivity [2, 3].

\*\* An analytical solution is obtained when the separation field, $E(\tau)$, is maintained at a constant value for a short period at the beginning of the separation, and then the field function is changed to an exponential form in a continuous manner.

$$E(\tau) = \begin{cases} v/S_{max}; & \{\tau \leq D/v\} \\ (v/S_{max})\exp(\tau v/D - 1); & \{\tau > D/v\} \end{cases}$$

If the separation field is held constant until the fastest band arrives at the detection zone, $S_{max}$ is the mobility parameter of the fastest band and $v$ is the velocity at which all bands are migrating when they arrive at the detection zone. In all cases, $D$ is constant and is the distance analytes must travel to reach the detection zone.

velocity can be achieved by increasing the field exponentially with time:

$$E(\tau) = \alpha \, \exp(\beta\tau) \, , \tag{2}$$

Here, $\alpha$ and $\beta$ are constants. This result is verified by calculating the time necessary for band $i$ to travel the distance, $D_{det}$, from the starting position to the detection zone and determining the velocity of the band at this time. This velocity must be independent of the electrophoretic mobility of the analyte, $\mu_i$.

For free solution electrophoresis performed in a capillary, the velocity of band $i$, $v_i$, is given by the vector sum of velocity arising from electrophoretic mobility and the velocity arising from electroosmotic flow:

$$v_i = \mu_i \, E + (-\varepsilon\zeta/\eta)E \tag{3}$$

$\varepsilon$ and $\eta$ are the solution permittivity and viscosity, respectively, $\zeta$ is the zeta potential of the capillary wall, and $E$ is the applied electric field. (For electrophoresis performed in gels or in other matrices that do not support electroosmotic flow, Eq. (3) reduces to $v_i = \mu_i E$). To simplify the notation, the quantity $(\mu_i - \varepsilon\zeta/\eta)$ can be represented by a single mobility parameter, $S_i$, so that

$$v_i = S_i E \tag{4}$$

From Eqs. (2) and (4), the velocity of species $i$ at time $\tau$ is

$$v_i(\tau) = S_i\alpha \, \exp(\beta\tau) \tag{5}$$

Therefore, the distance $d$ a band travels between times zero and $\tau$ is

$$d = \int_0^\tau v_i(\tau')d\tau' = S_i\alpha \int_0^\tau \exp(\beta\tau') \, d\tau' = (S_i\alpha/\beta)\exp(\beta\tau) - S_i\alpha/\beta \tag{6}$$

When $d = D_{det}$, $\tau$ becomes the elapsed time for species $i$ to reach the detection zone, $\tau_{det,i}$. Solving Eq. (6) for this quantity gives

$$\tau_{det,i} = (1/\beta)\ln[(\beta D_{det}/S_i\alpha) + 1] \tag{7}$$

Substitution of the result in Eq. (7) into Eq. (5) yields the velocity at which band $i$ passes through the detection zone:

$$v_{det,i} = \beta D_{det} + S_i\alpha \tag{8}$$

Equation (8) reveals that the velocity of an band at the detection zone is not dependent on the mobility parameter provided that $\beta D_{det} \gg S_i\alpha$. This condition can be met by the appropriate choice of $\alpha$, $\beta$ and $D_{det}$.

Figure 1 demonstrates the severe band compression incurred when the separation field is increased exponentially during capillary electrophoresis. In Fig. 1a, fluorescein-labeled arginine and fluorescein-labeled glutamate are the first and last bands, respectively, to reach the detection zone, and several fluorescein reagent bands are detected at intermediate times. The greater peak

width at later separation times is mostly attributable to slower analyte detection velocities (*i.e.*, diffusional broadening is a minor effect). Note that all bands are easily separated. In Fig. 1b an exponential field function is employed to separate the same species as in Fig. 1a. Here, the detection velocity for all bands is nearly identical but the final three peaks are compressed approximately to the limit of baseline resolution.

All analyte bands will pass through the detection zone at the same velocity with no band compression if both the electric field and the distance between the analyte starting point and the detection zone increase linearly with separation time:
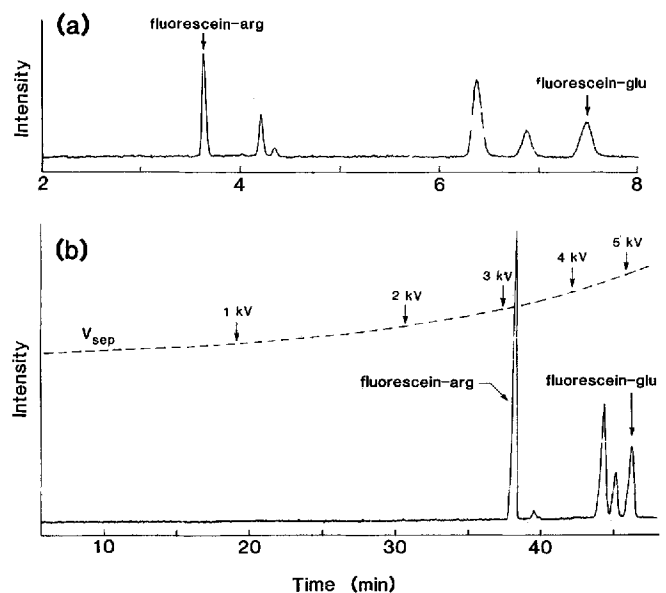
$$E(\tau) = E_o + \alpha\tau \tag{9}$$



*Figure 1.* Capillary electrophoretic separations of fluorescein-labeled amino acids demonstrating the compression of analyte bands that occurs when the separation field, $E(\tau)$, increases exponentially with time. (a) A separation using a constant applied field of 179 V/cm (10 kV) of fluorescein-arginine (nominally 2 nM) and fluorescein-glutamate (6 nM). The fluorescein reagent is responsible for the intermediate peaks. This separation allows one to calculate the mobility parameters for fluorescein-arginine and fluorescein-glutamate: $S_{fluor-arg} \approx 0.00037$ cm$^2$V$^{-1}$s$^{-1}$ and $S_{fluor-glu} \approx 0.00018$ cm$^2$V$^{-1}$s$^{-1}$. (b) A separation of fluorescein-arginine (20 nM) and fluorescein glutamate (20 nM), using a separation field (in V/cm) that increases exponentially with the separation time (in s), $E(t) = 5.45 \exp(0.001 \, \tau)$. When using this function, $\beta D_{det}$ is approximately ten times larger than $S_i\alpha$: [$S_{fluor-arg}\alpha = 0.002$ cm/s, $S_{fluor-glu}\alpha = 0.001$ cm/s, $\beta D_{det} = 0.0145$ cm/s]. The dashed line indicates the output of the power supply as the run proceeds. The baseline widths of the peaks are approximately equal, indicating that the bands pass through the detection zone at nearly identical velocities. Note, however, the dramatic compression of the peaks at late times. Experimental notes: The laser-induced fluorescence system employed in these separations is similar to that described previously (see [1]). The fused silica separation capillary (75 $\mu$m ID) has a total length of ~ 56 cm, and the distance from the inlet to the detection zone, $D$, is 14.5 cm. A 5 mM sodium phosphate buffer (pH 7.8) is employed as the separation electrolyte. The separation field is programmed using LabView 2 (National Instruments) for the Apple Macintosh, and a digital-to-analog converter (model NB-MIO-16XL-42, National Instruments) sends the controlling signal to a high voltage power supply (model 20/20, Trek, Inc.) which generates the separation voltage.

and

$$D(\tau) = D_o + \beta\tau \tag{10}$$

Again $\alpha$ and $\beta$ are constants. The velocity of species $i$ at separation time $\tau$ is

$$v_i(\tau) = S_i(E_o + \alpha\tau) \tag{11}$$

and the distance that species $i$ travels in time $\tau$ is

$$d = S_i \int_0^\tau (E_o + \alpha\tau') \, d\tau' = S_i\tau E_o + S_i\alpha\tau^2/2 \tag{12}$$

Species $i$ is detected at time $\tau_{det,i}$, at which point $d$ is equal to the distance from the starting point to the detection zone ($d(\tau_{det,i}) = D(\tau_{det,i})$). Consequently, Eq. (12) is equal to Eq. (10):

$$d(\tau_{det,i}) = S_i\tau_{det,i}E_o + S_i\alpha\tau^2_{det,i}/2 = D_o + \beta\tau_{det,i} \tag{13}$$

If $D_o$ and $E_o$ are both zero we can solve for the detection time of species $i$:

$$\tau_{det,i} = 2\beta/S_i\alpha \tag{14}$$

Equation (14) can be substituted into Eq. (11) to give the detection velocity of species $i$:

$$v_{det,i} = 2\beta \tag{15}$$

Equation (15) verifies that detection velocity is independent of electrophoretic mobility when the separation field and separation distance are increased linearly with separation time. Furthermore, because the detection time, $\tau_{det,i}$, scales inversely with the mobility parameter, no band compression should result from this procedure.

We demonstrated in earlier work that the effect of nonuniform analyte velocity on fluorescence sensitivity can be significant [1]. For an electrophoretic separation in which fluorescence sensitivity is optimized for a fast-traveling band, the SNR can range by more than a factor of five for bands arriving at the detection zone over a tenfold range in separation times. To attain uniform sensitivity at the highest possible SNR, either the detection system must be modified during the separation or analytes must be induced to travel through the detection zone at a uniform velocity. The problem of achieving uniform detection sensitivity during an electrophoretic separation is special to on-line detection. Clearly, the problem can be avoided by using off-line detection in

which the bands move through the detection zone at the same velocity. Although this approach is possible for capillary electrophoresis, off-line detection can be experimentally challenging; special care must be taken to ensure that band broadening does not occur as a result of capillary back-pressure [5] or from dead volume in miniaturized plumbing connections. Coupling of slab gels to post-separation flow cells may prove even more challenging. Recently, on-line detection for slab gel electrophoresis [6–8] has attracted significant attention. Real-time detection for slab gels eliminates manual analysis and abolishes the need to perform multiple, overlapping separations to view species with highly disparate electrophoretic mobilities. Because translation of a fluorescence detection zone is readily accomplished with slab gels [9], tube electrophoresis [10], and capillary electrophoresis [11], achieving a uniform analyte detection velocity is an attractive approach for maximizing fluorescence signal-to-noise ratios.

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