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Increasing the Efficiency of In-Capillary Electrophoretically Mediated Microanalysis Reactions via Rapid Polarity Switching

Brandi D. Sanders, **Rachel L. Slotcavage**, **Diana L. Scheerbaum**, **Christopher J. Kochansky**, and **Timothy G. Strein***
Department of Chemistry, Bucknell University, Lewisburg, Pennsylvania 17837

Abstract

We report herein a new approach to enhance the sensitivity or speed of CE-based methods that involve in-line reactions. Rapid polarity switching (RPS) is used as a novel means for in-line mixing of two reactant solutions via rapid (1–5 s) and sequential switching of the applied potential field. By employing the RPS approach with a model chemical reaction, that between creatinine and alkaline picrate, significant enhancement in sensitivity (or a decrease in analysis time) is realized. Both increased convection and electrophoretic stacking of the ionic reagent appear to contribute to the rise in apparent reaction rate. When coupled with in-line chemistry of the Jaffe method for creatinine, the RPS methodology allows for 3-fold faster determination of creatinine in the concentration range needed for the analysis of clinical blood serum specimens. The new approach also allows the analysis to be performed without the need for the cumbersome and problematic enhanced sensitivity cell.

Electrophoretically driven analysis systems, including those employing in-line chemical reactions, have a great deal to offer the bioanalytical chemist. Indeed, the great promise of microfluidic total analysis systems seems eminent. However, before any such assays become routine in, for example, clinical and medical laboratories, a good deal of investigation and optimization of methodology and sample handling procedures must be undertaken. For in-line chemical reactions, this means maximizing the effectiveness of in-line mixing of reagents, designing ways to stack the reagents, products, or both, and optimizing detection sensitivity to suit the analysis.

First reported in 1992 by Boa and Regnier,[1] the CE-based technique of electrophoretically mediated microanalysis (EMMA) involves the in-capillary mixing of very small volumes of solutions of two or more compounds based on differences in electrophoretic mobilities of the reactants. EMMA has numerous modes of operation,[2–4] but is commonly performed by injecting discrete “plugs” of reagent solutions into a capillary, an approach first reported by Whitesides.[5] The reagent with the slower mobility is first injected into the capillary followed directly by the injection of the second, higher mobility reagent. The electric potential is then applied across the capillary until the leading edge of the faster reagent “catches up” with the leading edge of the slower reagent, thereby mixing the zones of the two reagents. If necessary, the potential can be turned off once the reagent zones have overlapped to allow an increased product yield for reactions.[6,7]

Not surprisingly, most of the published data obtained with EMMA methodology involve enzymatic reactions.[1,5,8–16] When EMMA is carried out with enzymatic reactions, enzyme turnover can give rise to amplified amounts of product, which compensates for the characteristically poor concentration sensitivity with CE. EMMA can also be employed to carry out small-molecule chemistry,[17–24] but with these in-line reactions, detection sensitivity is a

* To whom correspondence should be addressed. E-mail:strein@bucknell.edu. Phone: 570-577-1541. Fax: 570-577-1739..

larger concern. In general, nonenzymatic systems have not been widely studied because of the difficulties posed by poor sensitivity. Indeed, we have encountered this situation in our work with EMMA for the clinically relevant chemistry of the Jaffe reaction (Figure 1) between creatinine and picrate.[25] While the method has proven useful for determination of creatinine in human sera, it requires the use of a cumbersome and costly, albeit commercially available, detection cell to produce accurate results with the lower end of the "normal" range of creatinine levels in serum.

In this article, we introduce the use of rapid polarity switching (RPS) as a way to improve the speed and sensitivity of EMMA with small molecules, using the Jaffe reaction as a model system. Rapid polarity switching, schematically shown in Figure 2, is a technique that takes advantage of the differences in electrophoretic mobilities of two reagents to produce successive mixing events through repetitive reversals of the applied potential. In addition to increased convection, if the ionic environments are favorable, it should be possible to invoke electrophoretic stacking of the ionic reactant plug(s), the product that ensues, or both. In these ways, RPS allows a scheme in which increased rates of reaction for the in-line chemistry can be achieved. In this paper, we introduce this methodology and explore the utility and limitations of this new approach.

THEORY

An important consideration when using the plug–plug EMMA technique (with or without RPS) is to ensure accurate reactant plug overlap. Accurate plug overlap is important because the greatest analytical response will be observed when the amount of time the mixing potential is applied results in complete overlap of the two reactant plugs.[1,6,7] Two parameters, the linear length of the reagent plugs (L_p), and the observed net mobilities (μ_{net}) of the reactants in each plug, must be considered to determine the appropriate time a given potential should be applied to ensure accurate plug overlap. The mathematical expressions that describe EMMA are well-understood, and a detailed theoretical treatment of EMMA can be found elsewhere.[6,7] Those equations critical to the success of this work are briefly described below.

The length of a "plug" of analyte solution as introduced by application of pressure can be calculated with the following equation:

$$L_p = \Delta P d^2 t_p / 32 L t \eta \quad (1)$$

where ΔP is the injection pressure (g/cm·s2), d is the inner diameter of the capillary (cm), t_p is the amount of time the injection pressure is applied (s), L_t is the total length of the capillary (cm), and η is the viscosity of the buffer (g/cm·s). The observed net mobility of the compounds in the plugs (μ_{net}) can be determined by

$$\mu_{net} = L_d L_t / t_m V \quad (2)$$

where L_d is the length of the capillary from injection to detection (cm), L_t is the total length of the capillary (cm), t_m is the migration time of the analyte (s), and V is the voltage applied to the capillary (V). The linear velocity (υ) of each of the reagents can then be calculated by

$$\upsilon = L_d / t_m \quad (3)$$

After finding the linear velocity for each of the reagents, the difference in linear velocity (Δυ) or the velocity of the reagents relative to each other can be found by the following equation:

$$\Delta \upsilon = \upsilon_{reagent1} - \upsilon_{reagent2} \quad (4)$$

where $\upsilon_{reagent\#}$ is the linear velocity of each of the reagents. The optimal amount of time for which the potential should be applied (t_v) can then be calculated by a simple equation:

$$t_v = L_p / \Delta \upsilon \quad (5)$$

where L_p is the length of the reactant plugs. These calculations are crucial in determining the appropriate amount of time the potential should be applied to allow for accurate overlap of the reagents. Following overlap, the potential is often turned off for an incubation period.

Rapid polarity switching, shown schematically in Figure 2, is performed by periodically reversing the polarity of the applied potential in order to move the reactant plugs back and forth relative to one another. Normal polarity in CE is defined as a positive potential being applied to the injection end of the capillary while the negative potential is applied to the detection end (step 3a). As described above, if a negatively charged sample is injected into the capillary ahead of a neutral sample and potential is applied at normal polarity, the two samples will mix (and eventually pass each other). If, however, as soon as the two samples are mixed, the polarity is switched, the samples will migrate in the opposite directions and separate again. If the polarity is then switched back, the two samples will mix again, and so on. Rapid polarity switching, it is thought, will allow many more dynamic mixing events and thus may produce more product, with the same overall time scale and amount of reagent.

EXPERIMENTAL SECTION

Reagents

Creatinine was purchased from Sigma Chemical Co. (St. Louis, MO). Picric acid (saturated aqueous solution, 1.2% w/v) was purchased from VWR Scientific (West Chester, PA). Boric acid was purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). Sodium hydroxide was purchased from Fisher Scientific (Fairlawn, NJ).

Standard Solutions

Borate buffer (20 mM) was prepared by dissolving boric acid in ~900 mL of 18 MΩ·cm water in a 1-L beaker. The pH was adjusted using 1 M NaOH and an Accument pH meter. The borate buffer was then transferred to a 1-L volumetric flask and properly diluted. A 47.2 mM picrate solution was prepared by mixing 10.00 mL of 1 M NaOH with 90.00 mL of 1.2% w/v (52.4 mM) picric acid (measured pH ~12.3). Standard creatinine solutions were prepared in 18 MΩ·cm water. Solutions of borate buffer and standard creatinine solutions were filtered through 0.2-μm PTFE membrane syringe filters into 1-mL polypropylene vials purchased from Hewlett-Packard (Palo Alto, CA) before use.

Serum

Human serum was obtained from Sigma Chemical Co. and deproteinized via ultracentrifugation with Centrifree filter tubes (Amicon Inc., Beverly, MA). To the tubes were added 450 μL of sera and 50 μL of either 18 MΩ water or an aqueous 5.00 mM creatinine standard. The filled tubes were spun in a Beckman J2-HS refrigerated centrifuge (Beckman Coulter, Fullerton, CA) at 3200 rpm (1600*g*) for 30 min. The filtrate was directly injected.

Equipment

An Agilent 3D capillary electrophoresis (Agilent Technologies, Palo Alto, CA) system with photodiode array UV/visible detection and Agilent Chemstations software were used for all separations. Extended light-path capillaries (Agilent Technologies) were used for all experiments.

Linear Velocity Determination

In order for the electrophoretic mixing to be effective, precise alignment of the reactant plugs is critical. CZE experiments to determine the linear velocity of the analytes, picrate and creatinine, were performed in the same capillary as all subsequent RPS experiments. A creatinine plug was first pressure injected into the capillary at 50.0 mbar for 3 s, followed directly by a picrate plug (50.0 mbar for 3.0 s). This injection sequence, while not necessary, avoids unwanted electrophoretic mixing of the two reagents, while allowing both reactants to be electrophoresed in the same run. Several separation voltages that spanned the range of the instrument's capabilities (5–30 kV) were applied in consecutive CZE runs, and the migration times were used to determine the electrophoretic mobilities and the relative linear velocities of each of the reactants. Borate buffer (20 mM, pH 9.00) was used, and detection at 210 and 485 nm was performed.

Traditional EMMA

Following the procedure of Kochansky et al.[25] EMMA was performed by sequential 3-s, 50-mbar hydrodynamic injections of picrate, creatinine, and borate buffer followed by the application of a mixing voltage for a duration of time such that the zones containing creatinine and picrate overlapped completely (according to the difference in linear velocities of the reactants for the applied voltage). The reaction proceeded with no applied electric field for 6 min, allowing for picrate and creatinine to react and form the Janovski product. Following the incubation period, a CZE separation of the reaction product from any unreacted creatinine and picrate was performed. The running buffer for all analysis was 20 mM borate, pH 9.0, and the capillary was maintained at a temperature of 25 °C. Detection was accomplished using diode array UV/visible detection monitoring absorbance at 210 and 485 nm. After each electrophoresis run, a 3-min flush with borate buffer (20 mM) was performed for capillary reequilibration.

Rapid Polarity Switching

RPS was carried out using the "injection table" functionality in the Chemstations software. The reactant solutions were injected as described above, followed by two steps that were repeated some number of times: (1) potential was applied across the capillary to electrophoretically align the picrate and creatinine plugs (as above), and (2) once the two plugs were aligned, the polarity of the applied potential was reversed, moving the reactant plugs apart again. This two-step process was repeatedly performed for the desired duration and was always ended with the picrate and creatinine plugs completely overlapped (as in step 3a in Figure 2). In some early experiments (data not shown), the picrate and creatinine reaction mixture was then allowed to incubate under no electric field (stopped flow). It should be stated that when the injection table functionality is used, the actual time line of RPS exceeds the programmed values. RPS can also be accomplished using the "time table" functionality, and the actual and programmed times agree; however, in the time table one can only program five RPS steps before exceeding the capability of the program. For up to five RPS events, the two RPS programming procedures give rise to equivalent quantitative data within experimental error.

RESULTS AND DISCUSSION

Shown in Figure 3 are representative electropherograms for the analysis of an aqueous creatinine standard with the in-line EMMA assay as carried out with no RPS. The electropherograms shown were collected (A) at 210 nm, where both reactants and the product can be visualized, and (B) at 485 nm, where the product absorbs strongly but neither reactant has a significant absorptivity. It is well known that this long-used and clinically accepted reaction does not proceed to completion,[25–28] and it is immediately evident from the data at 210 nm that this is the case with the EMMA approach as well.

By employing a simple but precisely controlled series of alternating potential pulses, the amount of Janovski product formed during the in-line reaction can be substantially increased. To investigate the effect of rapidly switching of the applied potential on the amount of product formed, we performed RPS for a given duration, while decreasing the post-RPS stopped-flow incubation time by the same amount of time. The amount of product observed was found to increase with the fraction of time that RPS was applied.

The effect of the RPS without a post-RPS incubation time is illustrated in Figure 4. As one might expect, the amount of product formed increases with the duration of RPS, and the initial rate of product formation exceeds that observed during successive events. Importantly, the RPS methodology can be employed to obtain roughly the same level of signal as obtained with a much longer stopped-flow experiment (Figure 5). In this case, 60 s of RPS at ±9.3 kV gave rise to nearly the same amount of product as was obtained with 6 min of stopped-flow reaction in the same capillary under the same conditions. In addition, with the RPS approach, peak widths are narrower than with a 6-min zero potential incubation. Certainly, diffusion leads to broadening of the analytes zones in both experiments, but with RPS, the total analysis time is lower; moreover, if the ionic constituents are favorable, as it appears they are in this case, tITP stacking during each RPS event could result in narrowing of the picrate or product zones.

To more fully characterize RPS, the effects of both the potential and the time of the RPS application were systematically considered. At higher RPS voltages, the difference in linear velocity ($\Delta\upsilon$) between creatinine and picrate will be greater, and therefore, a shorter duration of that potential is needed to align the two reactant zones. In the absence of detrimental effects owing to excessive Joule heating, the relationship between $\Delta\upsilon$ and the applied voltage is linear, allowing precise prediction of an acceptable set of potential and time parameters for RPS. For example, a linear plot ($R^2 = 0.9985$) was obtained over the range from 5 to 30 kV for the capillary used to collect the data shown in Figure 4 (50-μm i.d., 20 mM pH 9.0 borate buffer). For this capillary, with a reactant plug lengths of 1.8 mm (3 s, 50 mbar), the predicted mixing times for optimal alignment of the two plugs at four different RPS potentials is given in Table 1. It is noteworthy that the Agilent Chemstations software allows precision only to the nearest 0.1 kV and to the nearest 0.1 s (injections table) or 0.01 min (run time table), so judicious choice of RPS potential and time is required.

The effects of varying both the time and potential of RPS are illustrated in Figures 6 and 7. Interestingly, the amount of product formation depends on the voltage at which the RPS is performed, as well as the duration of RPS. The increase in signal is likely due to a number of factors, including (i) increased convection, (ii) a higher solution temperature owing to Joule heating at higher potentials, and (iii) electrophoretric stacking of the ionic picrate giving rise to higher local concentrations of this reagent. Convection and stacking are likely to be the primary factors. Any temperature increase appears to be minimal, as the observed currents at the potential(s) employed do not deviate significantly from typical operating conditions, which fall in the linear region of an Ohm's law plot for the system. Temperature gradients resulting from Joule heating was the subject of a recent review.[29] In this work, the use of a fairly low concentration (20 mM), low conductivity buffer system (borate) and 50-μm-i.d. capillary minimizes the severity of this unavoidable occurrence.

In Figure 6, the effects of four different RPS potentials are considered, and the time of RPS application is varied from 0 to 150 s, with product being monitored at 485 nm. This detection wavelength results in lower sensitivity than 210 nm, but allows a higher degree of specificity, as picrate and the other reagents used show minimal absorption at this wavelength. Product yield appears to increase with time as well as potential of RPS. However, decoupling these effects is complicated by the fact that changing RPS potential necessarily changes the time of each RPS step. That is, for a given duration of RPS, the number of RPS events increases with

increased voltage and linear velocity. Consider the data in Figure 6; an RPS mixing potential of 18.2 kV ($\Delta\upsilon$ = 1.00 mm/s) applied over a programmed duration of 28 s, gives rise to seven RPS mixing events, while nearly the same amount of time (26.4 s) of RPS at 5.8 kV ($\Delta\upsilon$ = 0.24 mm/s) results in only two RPS events. Thus, while the data in Figure 6 may appear to imply that higher RPS potentials give rise to higher product yield, it is not clear if the increased yield is a function of higher potential or of the higher number of RPS events.

To further investigate the factors giving rise to increased product formation, the RPS technique was performed at varied mixing potentials, and the area of the product peak was monitored as a function of the number of RPS mixing events (Figure 7). At first glance, these data may appear to indicate that, for a constant number of RPS events, higher potentials give rise to lower yield. However, one must consider that one RPS event is not the same duration at different potentials. It turns out that while the zones of the two reactants actually spend a greater amount of time in contact in the experiments employing lower voltages, the average rate of product formation is higher at higher RPS voltages.

Together, the results from Figures 6 and 7 indicate that the use of higher RPS potential gives rise to higher apparent reaction rate. The slight increase in linear velocity and, thus, the slight increase in collisional energy that would be expected from the use of higher potential seems inadequate, on its own, to explain the magnitude of observed increase in signal. It is possible that Joule heating raises the solution temperature, increasing the rate of reaction; however, the literature indicates that a higher temperature would result in decreased product yield.[30,31] This leads one to conclude that the increased reaction yield may be governed by an increase in local concentration of reactant(s) owing to stacking of picrate, the ionic reactant. Certainly there is also increased convection with more rapidly moving zones, but it appears as if stacking of the picrate at the “back” edge of the picrate plug also enhances product formation. Indeed, it is evident from the shape of the observed picrate peak that electrodispersion is occurring only in one direction, while electrokinetic stacking appears to be occurring at the opposite edge. Of course, it is expected that the picrate will be restacked at opposite edges of the picrate plug each time the potential is switched, and the highest concentration of picrate will be swept through the zone of creatinine. This situation is ideal as it results not only in successive restacking of the picrate with each RPS step but also in the relative movement of the most concentrated portion of the picrate zone through the creatinine zone with each RPS step. More detailed investigation of the effects of stacking during RPS are under way. Regardless, it is clear that the RPS methodology is successful in increasing product yield.

Importantly, the coupling of RPS with EMMA results in an increase in sensitivity that allows the direct determination of creatinine at concentrations typical in sera from healthy adults (0.1–0.3 mM), even with absorbance detection at 485 nm, without the necessity of a cumbersome and difficult to use high-sensitivity detection cell. If the RPS technique is to be analytically useful, the signal must also be indicative of the injected concentration (or amount) of creatinine. To show that RPS indeed accomplishes this goal, a calibration curve was constructed via analysis of creatinine concentrations from 0.1 to 20 mM. With a 65.0-cm-long capillary, 3-s 50-mbar injections, and 20 RPS events at 19.7 kV, the calibration curve exhibited good linearly ($y = 24.9x + 17$, $R^2 = 0.9982$) over a range of creatinine concentrations in aqueous standards from 0.10 to 20 mM. Based on a signal-to-noise ratio of 3, the lower limit of detection under these conditions was estimated to be 43 μM, and the RSDs for peak areas and migration times were uniformly less than 10% (n = 3) and 4% (n = 12), respectively. The analysis of two different batches of commercially available human sera by the method of standard addition resulted in product peaks that were wider and shorter and peak areas with, on average, larger RSDs (5–20%) than those obtained with the same creatinine concentrations in aqueous standards. Nonetheless, the RPS method resulted in quantitative data that fell in the expected range for sera from healthy patients (2.3 and 2.8 mg/dL). The differences in peak shape with

standards and serum specimens, indicates that additional sample preparation steps such as desalting may be necessary to fully realize the power of RPS with real samples and further underscores the likelihood that tITP stacking plays a significant role in the enhancement seen with RPS.

In general, for a given analysis time and sample, the RPS enhancement in sensitivity for the in-line chemistry considered here ranges from 50 to 400%, depending on the RPS potentials employed. Conversely, a 2–3-fold reduction in analysis time without loss of analytical signal can also be realized with RPS. It remains unclear how universal the RPS procedure may be for enhancing the rate of other in-line chemistry, and this is the subject of ongoing work. It seems likely that the procedure may be less useful for enzymatic systems, as the success might depend on the location of the binding site together with the directionality and conformation of the migrating enzyme.

CONCLUSIONS

Rapid polarity switching appears to be an effective way to improve reaction yield for in-line chemistry between picrate and creatinine. When carried out with careful attention to the injected plug size and the rate of electroosmotic flow, the RPS approach gives rise to reproducibly larger product peaks and accurate determination of creatinine using absorbance detection without the need for a special high sensitivity detection cell. Increasing RPS time or potential increases the amount of product formed, thus increasing the sensitivity of the technique. It appears likely that the advantages of RPS will extend to other chemistries as well, extending the applicability of the EMMA methodology to in-line reactions with slow kinetics without unduly increasing the analysis time.

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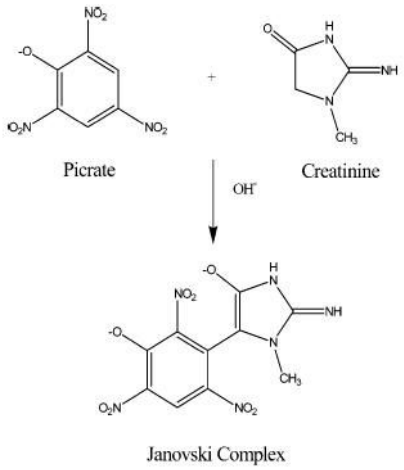

Figure 1.
Chemistry of the Jaffe reaction. Negatively charged alkaline picrate reacts with zwitterionic creatinine in the presence of sodium hydroxide to form the doubly anionic Janovski product, which is red in color and absorbs at ~500 nm.

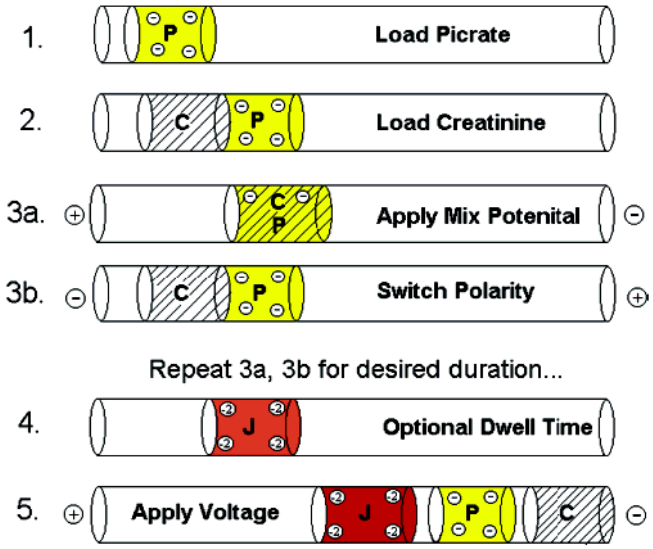

Figure 2.

Schematic of EMMA with rapid polarity switching. Steps 3a and 3b are repeated for the desired number of iterations or amount of time. The stopped-flow incubation time shown as step 4 is not performed in all experiments. Key: C = creatinine, P = picrate, J = Janovski complex.

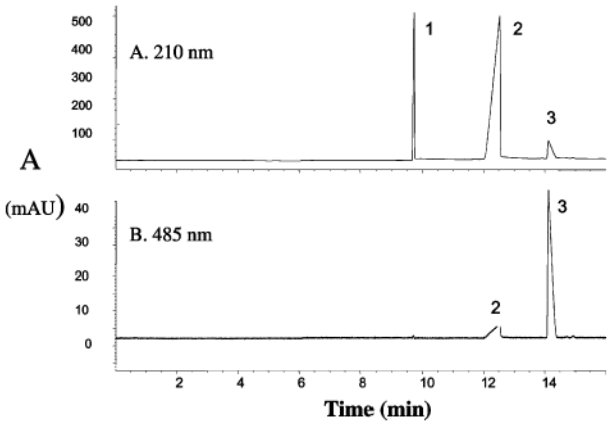

Figure 3.

Typical electropherogram for Jaffe chemistry performed with EMMA with no RPS. Data shown at (A) 210 and (B) 485 nm. Peak ID: 1, unreacted creatinine; 2, unreacted picrate; 3, Jaffe inline reaction product. EMMA conditions: 50-μm-i.d. extended light path capillary (L_t = 65 cm, L_d = 56.5 cm), 20 mM borate buffer (pH 9.00), 47.2 mM picrate (pH ~12.4), 20 mM creatinine, 300 mbar·s injections (1.8 mm), 20.9-kV overlap potential (1.8 s), 6-min zero potential reaction time, 27-kV separation potential.

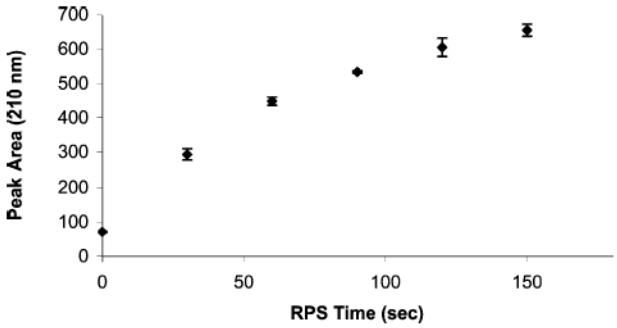

Figure 4.

Effect of duration of RPS on the amount of product formed during the in-line chemical reaction with no post-RPS dwell time. Separation conditions: 50-μm-i.d. extended light path capillary (L_t = 65.0 cm, L_d = 56.5 cm), 20 mM borate buffer (pH 9.00), 150 mbar·s injections of 47 mM picrate, 20 mM creatinine, 5.2-kV RPS voltage (7.5-s overlap time), 6-min total reaction time, 20.9-kV separation voltage.

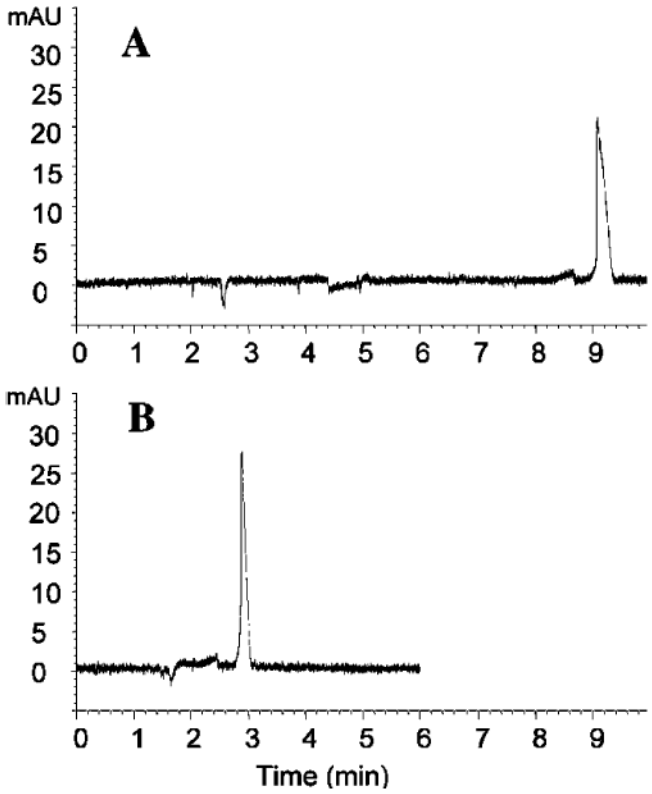

Figure 5.

EMMA electropherograms illustrating the capability of RPS. Shown are results from the in-line Jaffe chemistry with (A) normal one-step EMMA mixing and 6-min incubation and (B) 60 s of RPS with no incubation period. EMMA conditions: 50-μm-i.d. capillary (L_t = 50 cm, L_d = 41.5 cm), 20 mM borate buffer (pH 9.00), 150 mbar·s injections of 47.2 mM picrate (pH ~12.4) and 20 mM creatinine, 27-kV separation potential, detection at 485 nm. RPS at 9.3 kV (15 events, 4.0 s each). Note: No incubation time was employed for the data shown in viewbox B.

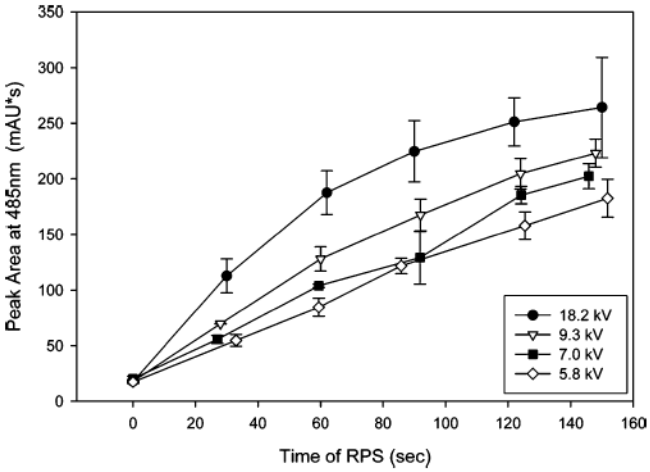

Figure 6.

Relationship between the time of RPS application and the product peak area at four different RPS voltages. Conditions: 50-μm-i.d. capillary (L_t = 50.0 cm, L_d = 41.5 cm), 3-s 50-mbar injection of 47.2 mM picrate solution and 20 mM creatinine standard; no post-RPS dwell time. Error bars represent standard deviation (n = 3).

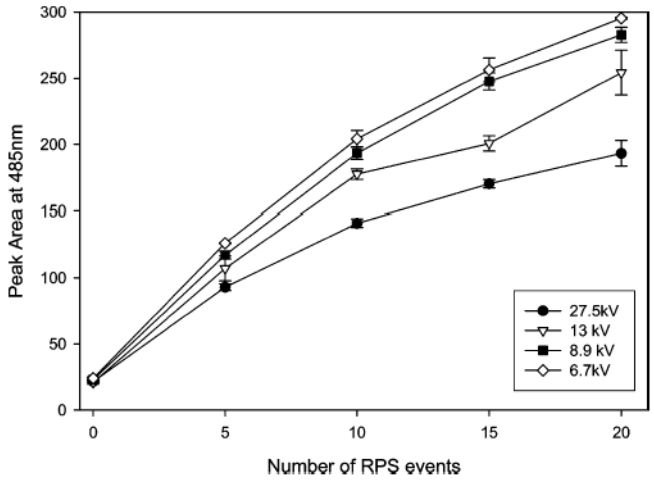

Figure 7.

Relationship between the number of RPS electrophoretic mixing events and product peak area at four different voltages. Conditions: 50-μm-i.d. capillary (L_t = 33.0 cm, L_d = 24.5 cm), 3-s 50-mbar injection of 47.2 mM picrate solution and 20 mM creatinine standard; no pos- RPS dwell time. Error bars represent standard deviation (n = 3).

Table 1

Predicted Time That a Given Potential Should Be Applied for Optimal Alignment of Adjacent 1.8-mm-Length Creatinine and Picrate Plugs in the Capillary Use To Collect the Data Shown in Figure 4

potential (kV)	calculated time for overlap (s)
5.2	7.5
12.6	3.0
15.8	2.3
20.9	1.8