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# Dynamic pH junction technique for on-line preconcentration of peptides in capillary electrophoresis

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**Abstract**

A method based on the presence of a dynamic pH junction within the capillary to induce band narrowing for enhanced detection sensitivity for some peptides is presented. This technique is predicated on a sharp reduction in an analyte's migration velocity following a reversal of its electrophoretic direction from the acidic sample zone to the basic BGS zone. Larger-than-usual injection volumes of samples in relatively high-conductivity matrices were enabled, without degrading peak shape, resolution and efficiency. The size of the original sample plug was reduced by as much as 38-fold, and improvement in detector response in terms of peak height by as much as 124-fold was obtained. The effects of pH and concentration of the sample matrix, and the length of sample injection on the efficiency of the technique are discussed.


*Keywords:* Dynamic pH junction; Peptides; Band narrowing

## 1. Introduction

Capillary electrophoresis (CE) is widely regarded to be superior to liquid chromatography (LC) in terms of efficiency. However, its application to routine analyses is often precluded by its poor concentration sensitivity, resulting from the use of capillaries with very small internal diameters (typically less than 100 μm), thereby limiting the amount of sample that can be loaded. Furthermore, since most commercial instruments are equipped with absorbance-related detectors, the problem is aggravated by the short path length available. While some efforts to improve detection limits have been directed at extending the path length (e.g. with the use of Z-cells or bubble cells) [1,2], most investigators placed emphasis in developing on-line preconcentration techniques, which enable injections of large volumes of sample without compromising resolution and efficiency [3–32]. By narrowing the analyte bands prior to their separation, signal enhancements ranging from a few tens to almost a million-fold could be achieved.

A very attractive feature of on-line preconcentration techniques is that they do not require any modification in existing instruments, since electrophoretic or chromatographic focusing of analytes is effected within the same capillary used for separation. Generally, they do so simply by exploiting a discontinuity between the sample matrix and the background solution (BGS), which causes an alteration in the analyte's mobility as it transits the boundary separating these two zones [27]. The discontinuity can be in terms of conductivity (e.g. in sample stacking)[3–8], the presence of an additive (e.g. micelle or complexing agent in sweeping) [9–17], salt content [18–20], or pH [21–32], among others.

A number of pH-mediated focusing strategies have already been developed, mostly for high salt-containing samples. In these strategies, the sample zone is titrated to neutral, creating a region of low conductivity where the analytes migrate quickly until they reach the boundary with the BGS and subsequently stack. Using pressure injections, Schwer and Lottspeich [21] sandwiched a solution of standard peptides between plugs of highly acidic and highly basic solutions. When voltage was applied, the $H^+$ and $OH^-$

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ions migrated toward each other converting the original sample zone into a low conductivity zone. The concept was extended to electrokinetic injection of cations from high conductivity matrices [22,23]. Sample injection was immediately followed by electrokinetic injection of a strong acid, which titrated the sample plug, forming a region of neutral charge and high resistivity through which the analytes could move rapidly. A parallel approach was employed for anions in physiological samples, this time with sequential electrokinetic injections of the sample and a strong base [24].

Changes in analyte velocity as a direct consequence of pH differences in multi-section capillaries have also been exploited as a means of focusing. In this case, the alteration in an analyte's net migration velocity is prompted by a change in its ionization state as it passes between two zones where a distinct pH difference exists. It was first demonstrated by Aebersold and Morrison using zwitterionic peptides [25]. Focusing was achieved by a reversal of charge and migration direction as anionic peptides in the basic sample zone entered the acidic milieu of the BGS. A recent modification by Wang et al. entailed a moderation of electrophoretic mobilities as the analytes reached the relatively more basic BGS zone [26]. The approach was extended by Britz-McKibbin et al. [27–30] to focus some weakly acidic compounds, and they used the phrase "dynamic pH junction" to describe the technique. In this case, the analytes were dissolved in a low pH solution and injected as a long plug into a capillary filled with a high pH BGS. When voltage was applied, a moving pH boundary swept through the sample zone. Analytes with weakly acidic functionalities were converted into their anions. Their net migration velocities decreased since they were now directed electrophoretically against the electroosmotic flow (EOF); hence, their bands were compressed into sharp zones. Kim et al. [31] employed an analogous technique to concentrate weakly basic compounds, this time using a very acidic sample matrix, where the analytes were cationic, and a mildly acidic BGS containing a cationic surfactant to reverse the EOF. Upon application of negative voltage, the analytes were hardly mobilized initially because their electrophoretic mobilities and the EOF were opposite each other. But once they have been titrated to neutral by the migrating BGS anions, they picked up speed, and were compressed into sharp bands. One key point about the dynamic pH junction technique is that it enables selective focusing of specific analytes by tuning the analytical conditions to favor such: given a sample matrix–BGS combination, only the analytes which experience an alteration in their ionization states significant enough to induce marked changes in their mobilities will be focused, while the others will largely remain unaffected; hence, they will migrate as broad bands. A better understanding of the underlying mechanism in this technique was obtained using computer simulation studies [32].

Peptides are rarely found in their uncharged states, and changes in local pH conditions could bring on large changes in their ionization; hence, a dynamic pH junction technique to focus them within the capillary is an attractive option. In this work, we present a simple method for preconcentrating peptides under highly alkaline conditions, according to a model depicted in Fig. 1. In brief, a plug of acidified sample is introduced into an uncoated capillary filled with basic BGS (A). When positive voltage is applied, the local pH at the right hand side of the sample plug–BGS interface rises sharply because of the interaction of the $H^+$ ions from the sample plug and $OH^-$ ions from the BGS. Fast-moving cationic peptides, upon reaching this juncture, are converted to anions, and they experience a drastic reduction in their migration velocities, since they must move now against a high, cathode-directed EOF. A pH moving boundary sweeps across the sample zone until the whole region is compressed (B), and the focused peaks separate and migrate to the detector (C).

## 2. Experimental

### 2.1. Apparatus

All CE experiments were performed on a Hewlett-Packard $^{3D}$CE System (Waldbronn, Germany) using fused silica capillaries (50 cm effective length × 50 μm I.D. × 360 μm O.D.) from Polymicro Technologies (Phoenix, AZ, USA), which were thermostated at 25 °C. The detection wavelength used was 200 nm. Water was purified with a Milli-Q system from Millipore (Bedford, MA, USA). Conductivities of samples and BGSs were measured using a Horiba ES-12 conductivity meter (Kyoto, Japan), while their pH values were measured and adjusted as required with the aid of a Mettler Toledo MP220 pH meter (Columbus, OH, USA).

### 2.2. Reagents and samples

Acetic acid, disodium hydrogenphosphate, methanol, sodium dihydrogenphosphate, sodium hydroxide, sodium borate, and thiourea were obtained from Wako (Osaka, Japan). Ammonia solution was from Nacalai Tesque (Kyoto, Japan). The proteins and peptides were from Sigma (St. Louis, MO, USA). All reagents were of analytical grade and used without further purification. All solutions were degassed and passed through 0.45 μm filters (Nacalai Tesque) prior to use.

### 2.3. General electrophoresis procedure

A new capillary was conditioned by successive rinsing with 1 M NaOH (20 min), methanol (20 min), and water (20 min). To ensure reproducibility between consecutive analyses, the capillary was flushed with 1 M NaOH (3 min), methanol (3 min), and water (3) before filling with the BGS (5 min). All rinses were carried out at ca. 1 bar, while sample injection was at 50 mbar. To approximate the length of injected zones, the velocity of a liquid in the capillary at

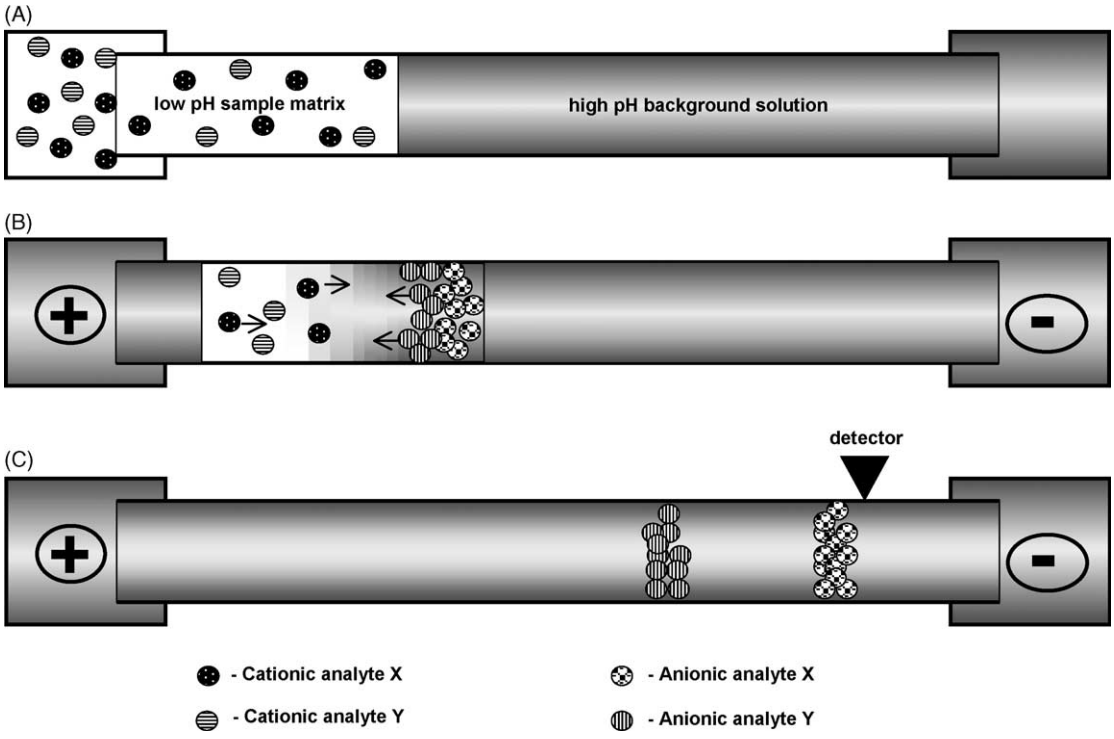


Fig. 1. A dynamic pH junction model for acidic peptides and proteins. (A) A long plug of sample in a low pH matrix is injected into an uncoated capillary filled with a high pH BGS. (B) A steep pH boundary develops at the front end of the plug and sweeps throughout the sample zone during electrophoresis, converting the cationic analyte into anionic and significantly retarding its migration velocity. (C) The focused peak migrates to the detector.

50 mbar was determined using a neutral marker. The measured flow rate was then multiplied by the injection time to give the length of the injected sample.

## 3. Results and discussion

### 3.1. Effect of sample matrix pH

Four peptides, with isoelectric points (p*I* values) which covered a fairly wide range were chosen as models for this study. These were bradykinin (RPPGFSPFR, p*I* 12.00), angiotensin I (DRVYIHPFHL, p*I* 6.92), angiotensin II (DRVYIHPF, p*I* 6.74), and [Sar$^1$, Ile$^8$]-angiotensin II (Sar-RVYIHPI, p*I* 8.75). The p*I* values indicated here were computed using http://kr.expasy.org/tools/pi_tool.html. It must be noted, however, that for [Sar$^1$, Ile$^8$]-angiotensin II, glycine was substituted for sarcosine (*N*-methylglycine, a non-standard amino acid) for computational purpose. Fig. 2A depicts their separation using 200 mM borate at pH 11. To examine the effect of pH of the sample matrix on focusing, portions of the test mixture were diluted with acetic acid solutions, the pH values of which were varied between 3.5 and 4.5 in increments of 0.25 unit, and the diluted solutions were injected into the capillary for 100 s (8.2 cm plugs). The resulting electropherograms are shown in Fig. 2B–F. As expected, the strongly basic peptide bradykinin (peak 1, p*I* 12.00) concentrated poorly because it was cationic in both the sample and the BGS, while angiotensin I (peak 3, p*I* 6.92) and angiotensin II (peak 4, p*I* 6.74) were reasonably well-focused within the range studied. The basic peptide [Sar$^1$, Ile$^8$]-angiotensin II (peak 2, p*I* 8.75) was more susceptible to changes in the pH of the matrix. Its focusing was good at pH 3.75, but deteriorated thereafter. Although sharp peaks for angiotensins I and II were also obtained at pH 3.75, they were not as well separated as in slightly higher pH conditions. At pH 3.5, the peaks were not resolved at all, and a broad peak corresponding to acetic acid [32] (marked with * in Fig. 2B) was detected early.

Obviously, focusing could not be effected by simply using a sample matrix with a pH value lower than the p*I* values of the peptides. Spectrophotometric experiments revealed that the actual initial pH of the sample zone is higher than expected, probably due to mixing at the sample–BGS boundary [32]. Since focusing is initiated by a sharp pH change generated at the interface, and which subsequently spreads throughout the sample region, a marked pH difference between the two zones is advantageous. Therefore, the effective initial pH of the sample matrix should be sufficiently low. This is evident in the case of [Sar$^1$, Ile$^8$]-angiotensin II, which required pH 3.75 in the sample matrix to preconcentrate efficiently. However, optimum band narrowing is not guaranteed either by the largest pH difference between the BGS and the sample. As shown in Fig. 2, slightly better results for angiotensins I and II were obtained using pH 4.25, and not at lower pH values. When the acidity of the sample matrix was too high, the positive charges on the peptides

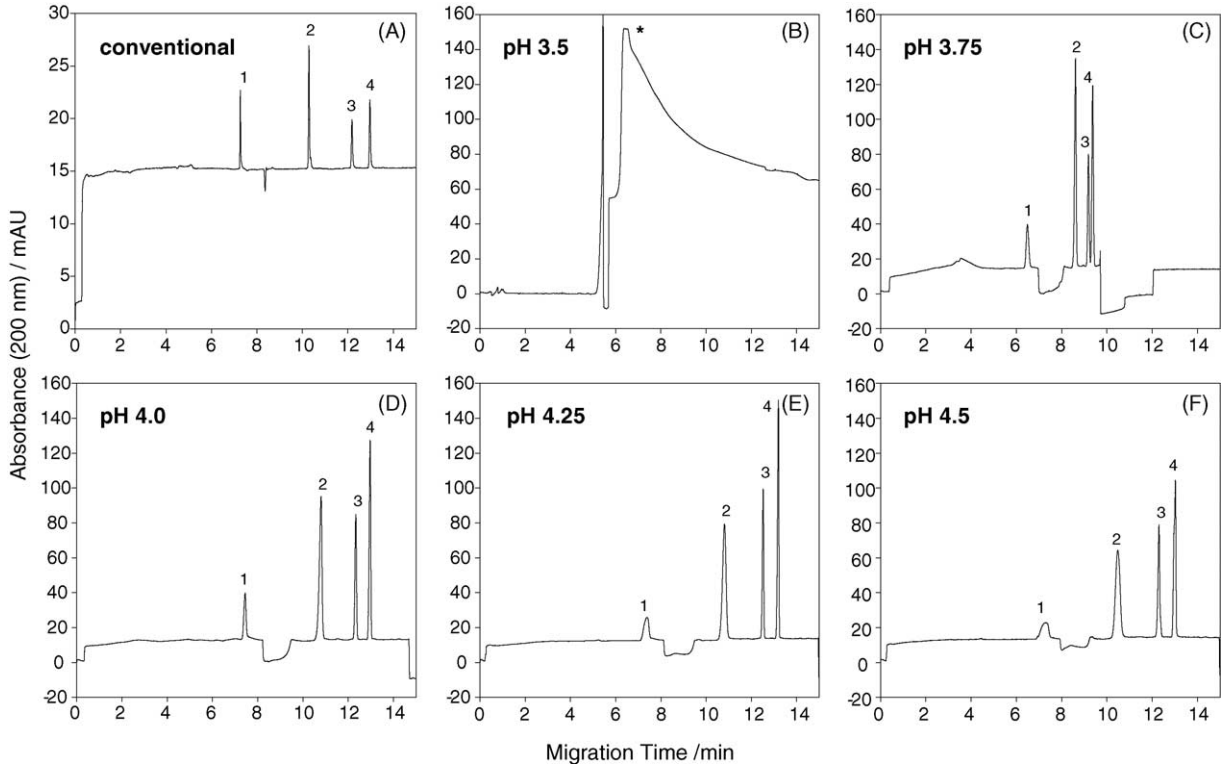


Fig. 2. Effect of sample matrix pH on focusing. Conditions: BGS, 200 mM borate, pH 11.0; sample matrix, 350 mM acetic acid with pH varied as shown; injection, 1 s of a mixture containing 100 ppm of each peptide (A), 100 s of a mixture containing 10 ppm of each peptide (B–F); applied voltage, +15 kV; detection, 200 nm. Peak identification, bradykinin (1), [Sar$^1$, Ile$^8$]-angiotensin II (2), angiotensin I (3), angiotensin II (4). Other conditions are given in the Section 2.

could not be sufficiently neutralized in the course of electrophoresis. Therefore, the optimum BGS–sample combination in terms of pH is one which can cause the largest change in the analyte's ionization state, and consequently, in its mobility.

Fig. 3 shows plots [33] of the peptides' net charges against pH to illustrate the changes in their ionization states. For purpose of comparison, a measure of the change in effective charge between pH 4.0 and pH 11.0 is marked in each plot, although these conditions are not strictly met for reasons described previously. The change in bradykinin is approximately 1 unit only, and it remains positive within the range under consideration. In contrast, [Sar$^1$, Ile$^8$]-angiotensin II, angiotensins I and II have been changed by approximately 3, 5 and 4 units, respectively. All three undergo transition from cation to anion. A more pronounced change in an analyte's effective charge should translate to a greater change in its electrophoretic mobility, and under these conditions, to a more abrupt reduction in its migration velocity, and possibly, to enhanced focusing.

### 3.2. Effect of injection length

By some electrophoretic or chromatographic mechanism, an on-line preconcentration technique decreases the width of the injected sample band, so that there is no breakdown in resolution nor efficiency despite the larger-than-usual injection volumes. The injection is prolonged until there is no more significant increase in detector response over the previous shorter injection, or the peak shapes and resolution begin to deteriorate. A convenient way to evaluate its performance is by comparing peak heights, and expressing the effectiveness as an $x$-fold improvement in response using the on-line preconcentration technique over a typical injection. Alternatively, it can be assessed by considering the extent to which the length of the original sample plug is reduced, i.e., by getting the ratio between the bandwidths at detection ($W_{\text{detection}}$) and injection ($W_{\text{injection}}$) points, or the detector-to-injection bandwidth ratio (DIBR) [27]. An even better way is to compare the standard deviations of the widths, since this will take into account the difference in shapes of the bands: the initial sample plug is assumed to be rectangular, whereas the detected peak is Gaussian. For the purpose of simplicity, however, the bandwidths are compared. Bandwidths are used because, unlike peak widths, they are independent of migration rates [34]. Generally, the sample band becomes broader as it migrates across the capillary because of diffusion and other dispersive effects, these being more evident in the case of late-migrating analytes. In contrast, no substantial broadening ensues or the sample band becomes even much shorter if a focusing mechanism is

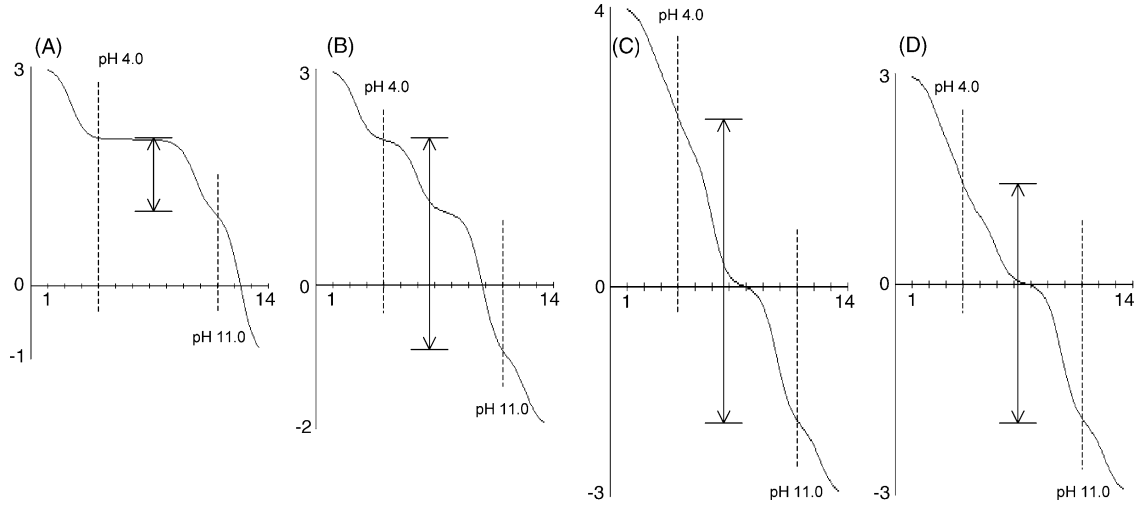


Fig. 3. Titration curves [33] of the model peptides. Bradykinin (A), [Sar[1], Ile[8]]-angiotensin II (B), angiotensin I (C), and angiotensin II (D). Note that for (B), glycine was substituted for sarcosine.

used [27]. Hence, the DIBR is an obvious measure of zone compression.

Fig. 4 graphs DIBR against the injection bandwidth using sample matrices with pH 4.0 (A) and 4.25 (B). It may be inferred that, up to a certain point, band narrowing became better as the injection length increased, producing much sharper peaks. These results were consistent with previous findings, and thought to be due to the formation of a slow moving, yet steep pH boundary [32]. Beyond a limit, however, the efficiency of band narrowing no longer improved, as a consequence of overloading. This was not immediately apparent if only peak heights were tracked since these were still increasing, albeit the increases were not better compared to the preceding shorter injection. Slightly better results were obtained using pH 4.0, but peak resolution was worse. Beyond 120 s (9.84 cm), peaks 3 and 4 overlapped.

### 3.3. Effect of sample matrix concentration

Since the dynamic pH junction technique involves titration of the sample zone, another factor which clearly influences its efficiency is the concentration of the sample matrix. The effect of the concentration of acetic acid, pH 4.0, on bandwidths at detection point was investigated in the range from 200 to 1300 mM, as shown in Fig. 5A. The injection time was kept at 60 s (4.92 cm). A plot of the concentration of

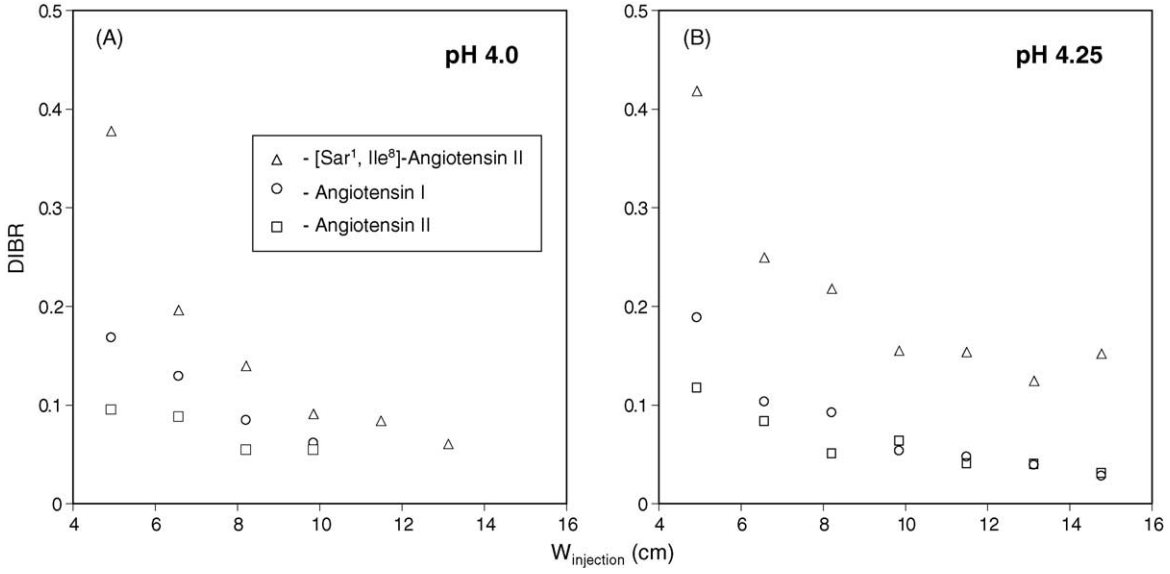


Fig. 4. Effect of injection length on band narrowing. Conditions: sample matrix, 350 mM acetic acid, pH 4.0 (A), and pH 4.25 (B). Other conditions are the same as given in Fig. 2.

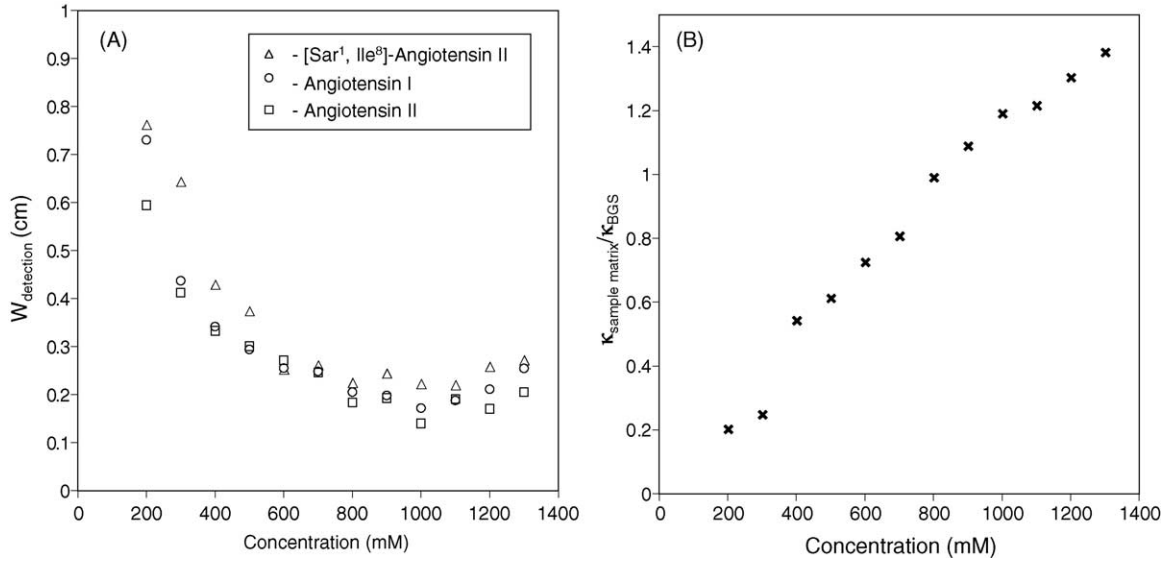


Fig. 5. Effect of sample matrix concentration on detection bandwidths (A), and ratio of the conductivity of the sample matrix with that of the BGS (B). Conditions: sample matrix, pH 4.0, with concentrations varied as shown; injection, 60 s. Other conditions are the same as given in Fig. 2.

the sample matrix against the ratio of the conductivities of the sample matrix ($\kappa_{\text{sample matrix}}$) and the BGS ($\kappa_{\text{BGS}}$) is also shown in Fig. 5B.

Bandwidths progressively became narrower by increasing the concentration of acetic acid in the sample matrix until 1000 mM. The focusing mechanism was evidently independent of conductivity-based stacking, as the results even improved by increasing the conductivity of the sample matrix. The peaks were narrowest when 1000 mM acetic acid was used, in which case, the conductivity of the sample matrix was approximately 20% higher than that of the BGS. At higher concentrations, the peaks broadened gradually. Similar to the impact of very low sample matrix pH, this could be attributed to incomplete titration. The concentration of $H^+$ was too high for the $OH^-$ from the BGS; hence, the induced changes in ionization and mobility were less striking compared to the previous lower $H^+$ concentration.

### 3.4. Focusing under optimum conditions

To focus one weakly basic ([Sar$^1$, Ile$^8$]-angiotensin II) and two acidic model peptides (angiotensins I and II) using the set conditions for dynamic pH junction, the most favorable sample matrix was 1000 mM acetic acid, pH 4.0. The injection was kept at a moderately long 60 s (4.92 cm). A typical electropherogram resulting from such conditions is depicted in Fig. 6. Table 1 summarizes reproducibility data on some peak parameters, band narrowing factors, and sensitivity enhancement factors in terms of peak height. As much as 38-fold reduction in length of the original sample zone, and 124-fold increase in detector response were achieved.

Preconcentration of the strongly basic peptide bradykinin was not successful under similar conditions. However, using a reverse pH junction technique (i.e., high pH sample matrix, low pH BGS), good focusing was attained (data not shown).

### 3.5. Mass balance

As a consequence of overloading, no significant increase in peak heights can observed, and peak shapes begin to deteriorate beyond an optimum injection length. In contrast, peak areas continue to increase, since these are directly related to the amount of sample introduced into the capillary. To ensure mass balance, the peak areas of the analytes injected under non-focusing and focusing conditions were compared. The results are shown in Table 2. The values obtained were fairly consistent, suggesting good recovery, i.e., no material loss under focusing conditions.

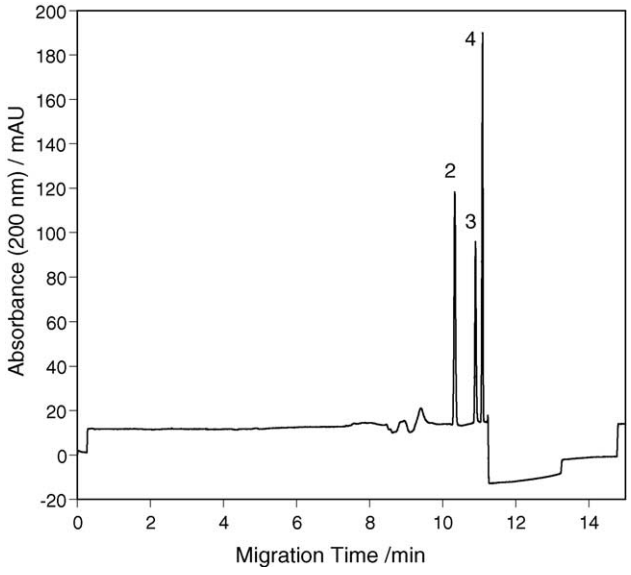


Fig. 6. Optimum focusing of the model peptides. Conditions: sample matrix, 1000 mM acetic acid, pH 4.0; injection, 60 s. Other conditions are the same as given in Fig. 2.

Table 1
Relative standard deviations (RSDs), band narrowing factors (BNFs), and sensitivity enhancement factors ($SEF_{height}$)[a]

| | Analyte | | |
|---|---|---|---|
| | [Sar[1], Ile[8]]-Angiotensin II | Angiotensin I | Angiotensin II |
| RSD (%, $n = 3$) | | | |
| Migration time | 0.4 | 0.4 | 0.5 |
| Corrected peak area[b] | 5.8 | 4.5 | 6.9 |
| Peak height | 11.0 | 5.3 | 7.6 |
| BNF[c] | 19 | 26 | 38 |
| $SEF_{height}$[d] | 65 | 113 | 124 |

[a] Conditions are the same as given in Fig. 6.
[b] Peak area/migration time.
[c] (1/DIBR); $W_{injection}/W_{detection}$.
[d] $SEF_{height}$ = (height with dynamic pH junction/height with conventional injection) × dilution factor.

Table 2
Comparison of peak areas with and without preconcentration[a]

| Analyte | Without preconcentration | With preconcentration[b] | |
|---|---|---|---|
| | 10 ppm | 10 ppm | 1 ppm |
| [Sar[1], Ile[8]]-Angiotensin II | 148.8 | 141.7 | 15.7 |
| Angiotensin I | 96.8 | 91.3 | 9.4 |
| Angiotensin II | 212.0 | 210.7 | 23.9 |

[a] The values are shown as the means of three runs using 60-s injections.
[b] Conditions are the same as given in Fig. 6.

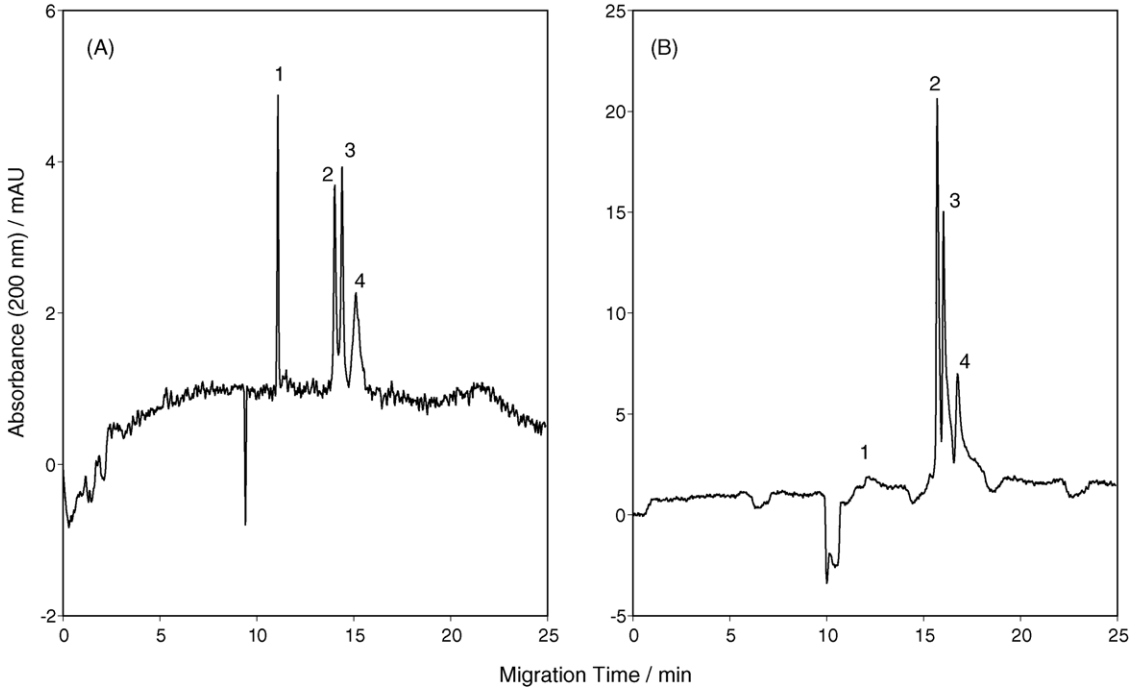


Fig. 7. Focusing of some acidic proteins by dynamic pH junction. Conditions: BGS, 50 mM sodium phosphate, pH 9.8; 50 mM sodium phosphate, pH 2.5; injection, 1 s of a mixture containing 200 ppm of each component in water (A), and 60 s of a mixture, diluted with 50 mM sodium phosphate, pH 2.5, containing 20 ppm of each component. Peak identification, myoglobin (1), β-lactoglobulin B (2), β-lactoglobulin A (3), and bovine serum albumin (4). Other conditions are the same as given in Fig. 2.

### 3.6. Application to protein analysis

To extend the usefulness of the technique, it was applied to the analysis of some acidic proteins as well. Fig. 7 compares a standard 1-s injection of a mixture containing 200 ppm of each protein with that of a 60-s injection of a 10-fold diluted mixture under dynamic pH junction conditions. In preliminary experiments, despite the strong acidity of the sample matrix, myoglobin, (peak 1) did not focus at all. Variants B (peak 2) and A (peak 3) of β-lactoglobulin focused to an extent of 67- and 43-fold, respectively. The peak shape of bovine serum albumin (peak 4) was rather poor making measurements difficult. Further studies on how proteins focus under similar conditions are currently underway.

## 4. Conclusion

We have shown a facile way of improving concentration sensitivity in CE for zwitterionic compounds by utilizing a pH junction. The method can tolerate the use of relatively high-conductivity matrices, which underscores its potential for the analyses of real samples. Knowledge of how the ionization changes with pH should aid in the rational design of experimental conditions, such as pH, composition and strength of sample matrices and BGSs, for efficient, selective focusing of some analytes.

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