Investigation of the Linearity and Stability of the pH Gradient Formed by Carrier Ampholytes with Whole Column Detection cIEF

Tiemin Huang, Jiaqi Wu, Convergent Bioscience Ltd., Toronto, ON, CANADA M8Z 2L8

INTRODUCTION

Isoelectric focusing (IEF) is the highest charge based resolution separation technique for proteins. It is commonly known that the pH gradient formed by synthetic carrier ampholytes (CAs) in slab gel IEF is linear, but it is not stable with a prolonged focusing time. The linearity and stability of the CAs' pH gradient in cIEF were investigated by observing the dynamic focusing of 4 commercial carrier ampholytes and the pH linearity at different focusing times using whole column detection cIEF (the iCE280 IEF Analyzer) and a set of pI markers (pI 3.21, 3.59, 4.22,4.65, 5.12, 5.85, 6.14, 6.61, 7.05, 7.40, 7.65, 8.18, 8.40, 8.79, 9.22, 9.46 and 9.77).

Figure 1a. Dynamic Focusing of pI makers in pH 3.5-9.5 Ampholine

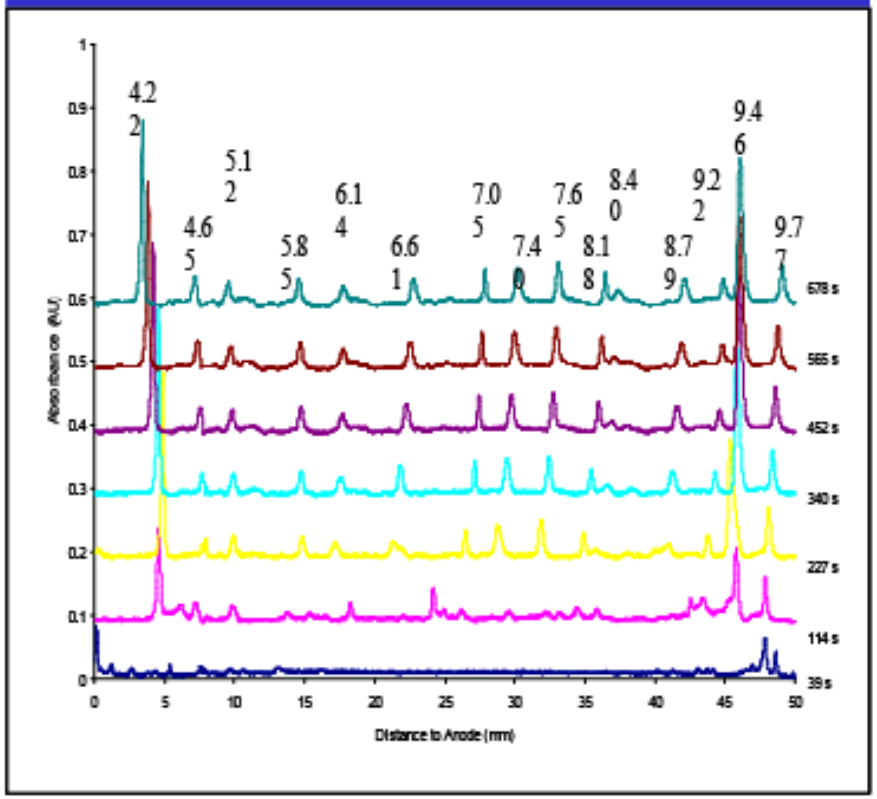
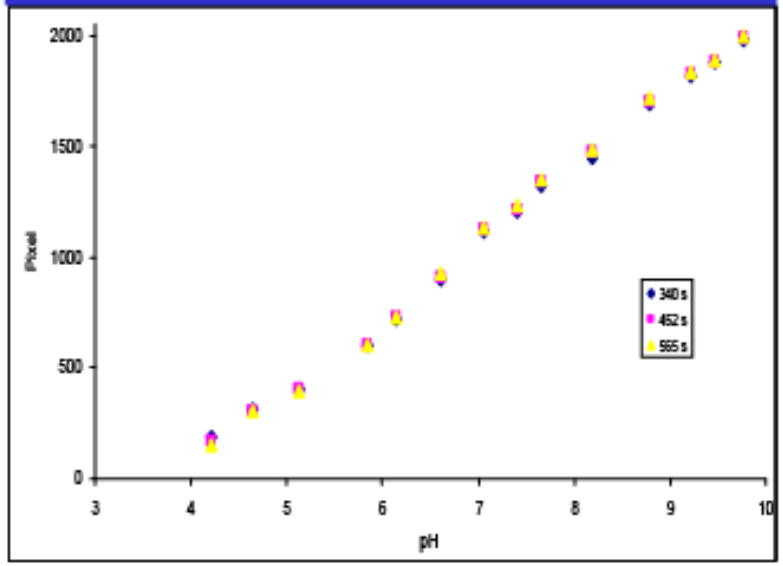


Figure 1b. Linearity of pH 3.5 9.5 Ampholine at Different Focusing Time



Experimental Conditions: pI markers were mixed with 8% (V/V) pH 3.5-9.5 Ampholine and 0.35% (W/V) methyl cellulose. Focusing voltage was 60/mm. The e-grams at different focusing time were overlaid (labeled at the right).

Figure 2a. Dynamic Focusing of pI makers in 8 M Urea and pH 3.5-9.5 Ampholine

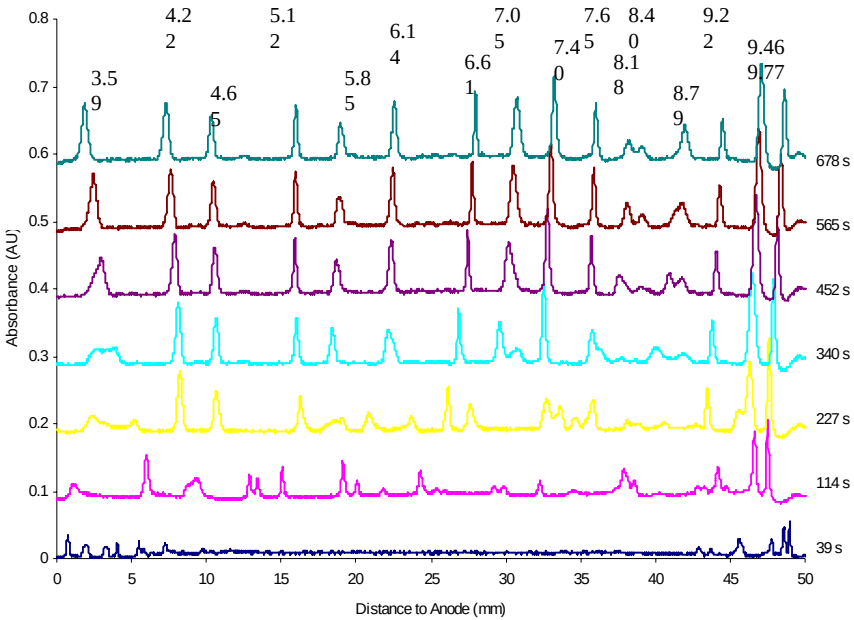
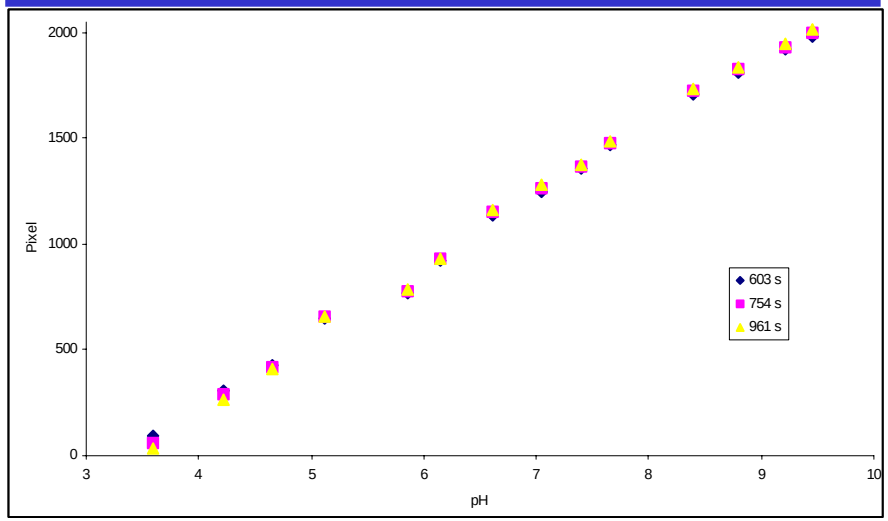


Figure 2b. Linearity of pH 3.5-9.5 Ampholine in 8 M Urea at Different Focusing Times



Experimental Conditions: pI markers were mixed with 8 M Urea, 8% (V/V) pH 3.5-9.5 Ampholine and 0.35% (W/V) methyl cellulose. Focusing voltage was 60V/mm. The e-grams at different focusing time were overlapped (labeled at the right).

Figure 3a. Dynamic Focusing of pI makers

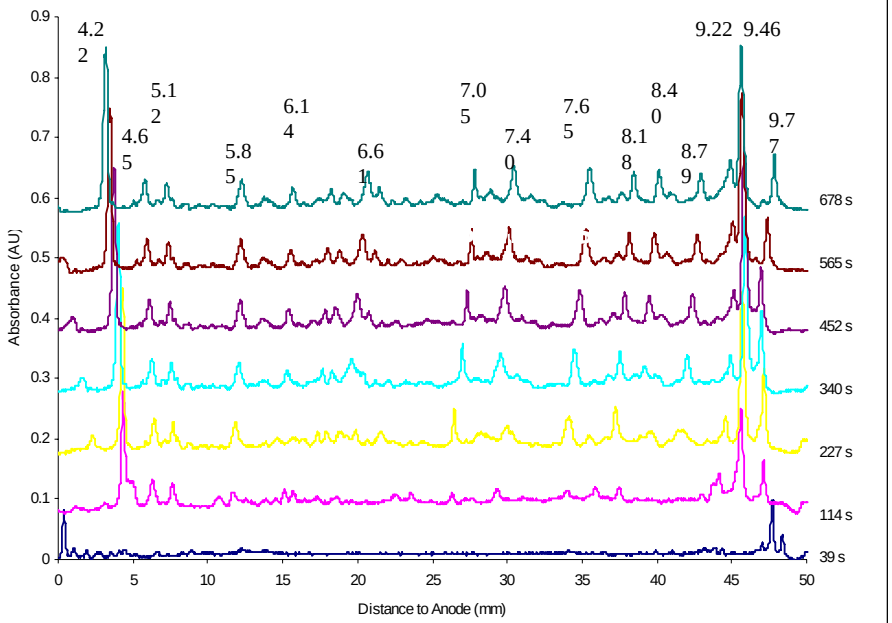
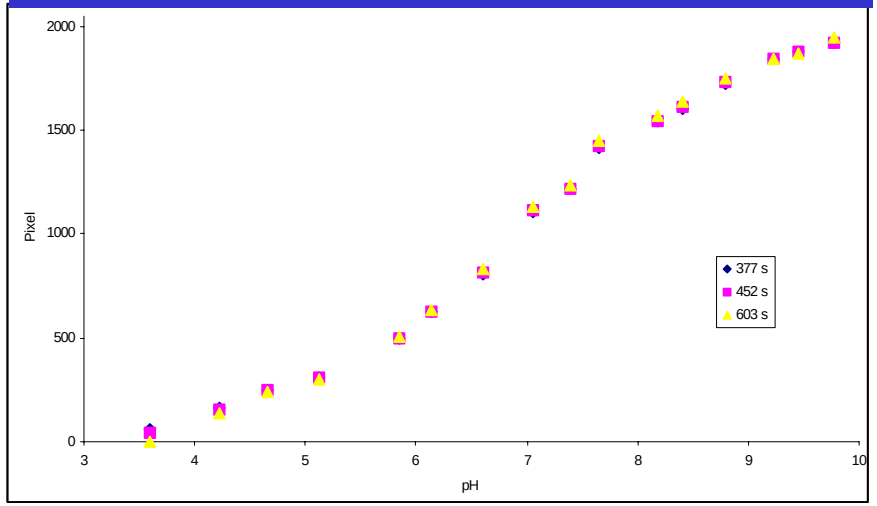


Figure 3b. Linearity of pH 3-10 Biolytes at Different Focusing Times



Experimental Conditions: pI markers were mixed with 8% (V/V) pH 3-10 BioLytes and 0.35% (W/V) methyl cellulose. Focusing voltage was 60V/mm. The e-grams at different focusing time were overlapped (labeled at the right).

Figure 4a. Dynamic Focusing of pI makers in 8 M Urea and pH 3-10 Biolytes

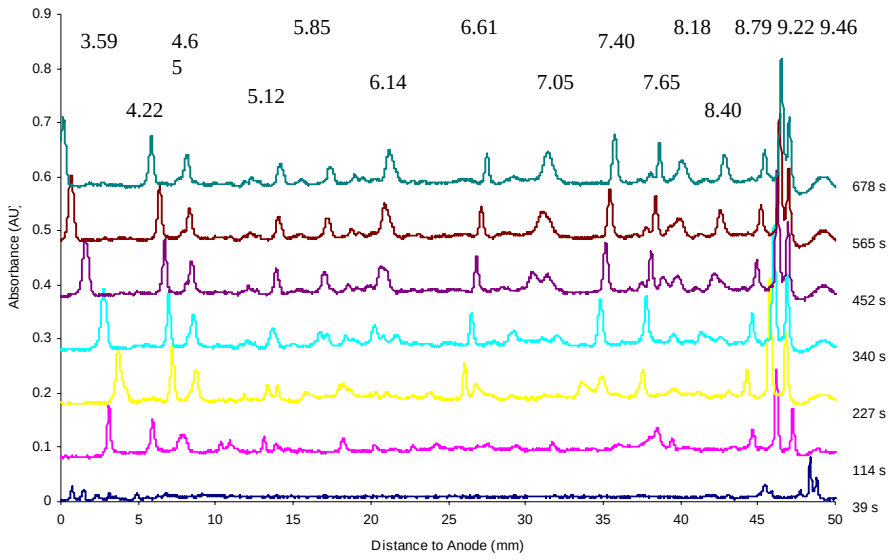
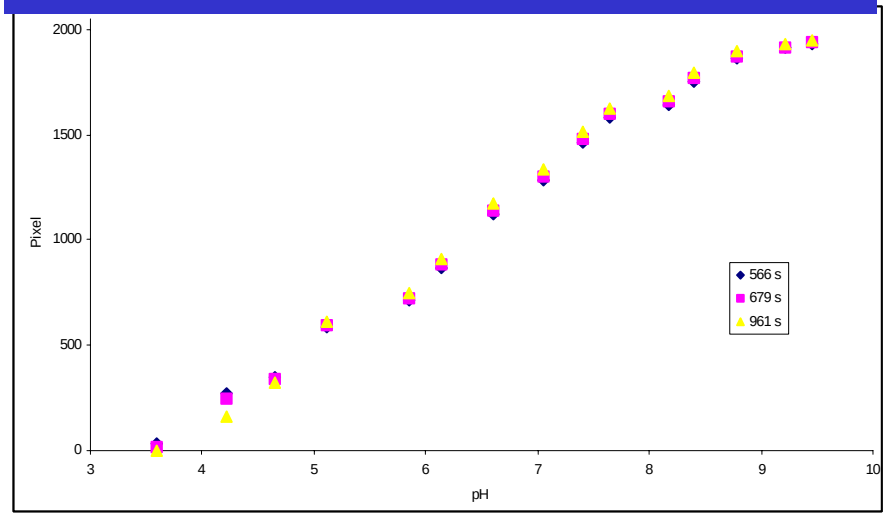


Figure 4b. Linearity of pH 3-10 Biolytes in 8 M Urea at Different Focusing Times



Experimental Conditions: pI markers were mixed with 8 M Urea, 8% (V/V) pH 3-10 Biolytes and 0.35% (W/V) methyl cellulose. Focusing voltage was 60V/mm. The e-grams at different focusing times were overlapped (labeled to the right).

Figure 5a. Dynamic Focusing of pI Makers in pH 3-10 Pharmalytes

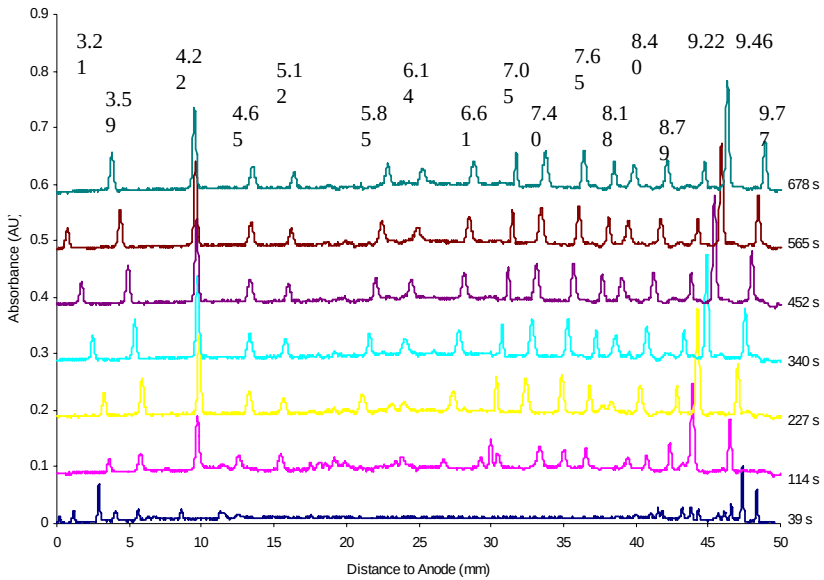
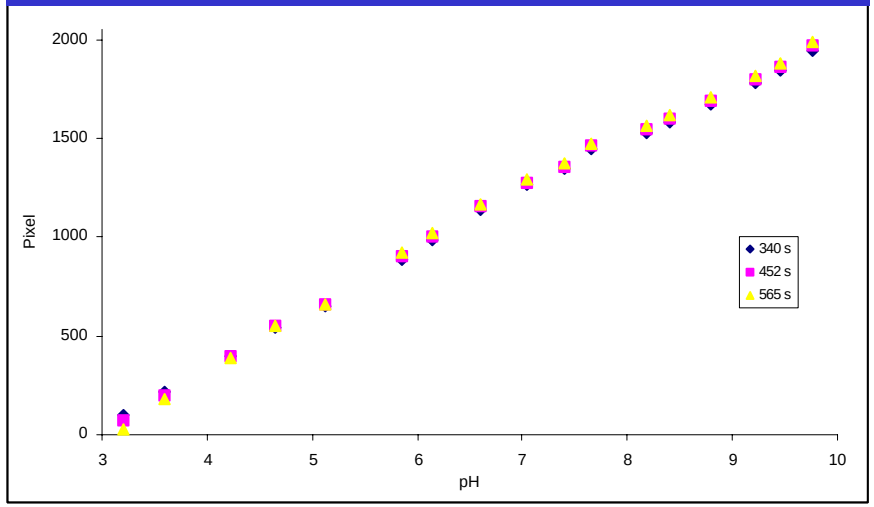


Figure 5b. Linearity of pH 3-10 Pharmalytes at Different Focusing Time



Experimental Conditions: pI markers were mixed with 8% (V/V) pH 3-10 Pharmalytes and 0.35% (W/V) methyl cellulose. Focusing voltage was 60V/mm. The e-grams at different focusing times were overlapped (labeled at the right).

Figure 6a. Dynamic Focusing of pI makers in 8 M Urea and pH 3-10 Pharmalytes.

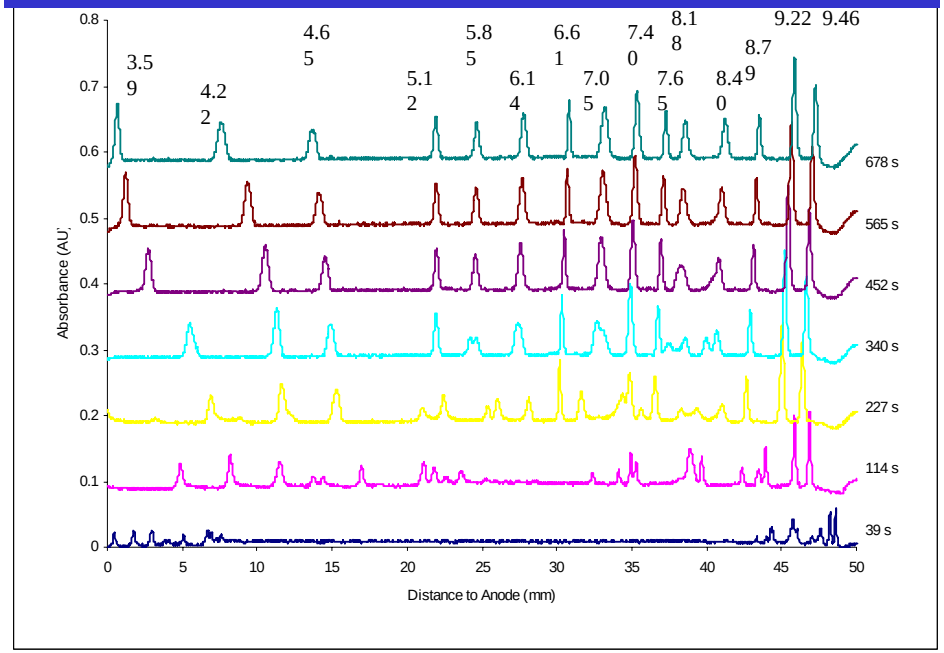
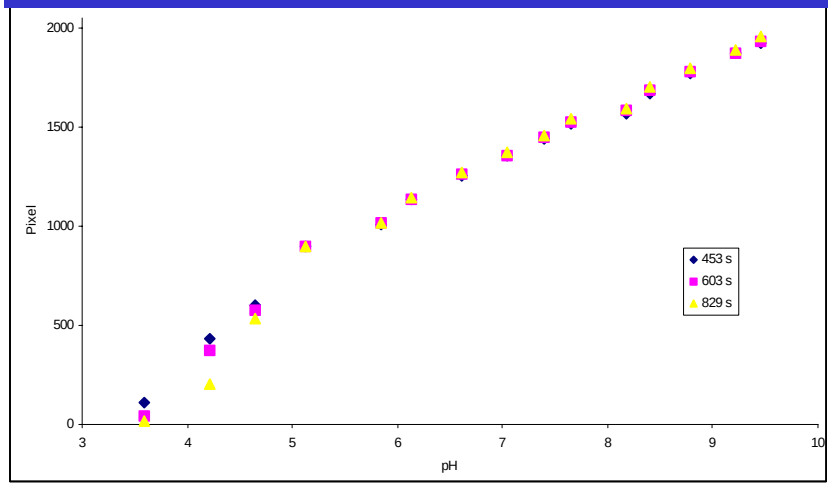


Figure 6b. Linearity of pH 3-10 Pharmalytes in 8 M Urea at Different Focusing Time



Experimental Conditions: pI markers were mixed with 8 M Urea, 8% (V/V) pH 3-10 Pharmalytes and 0.35% (W/V) methyl cellulose. Focusing voltage was 60V/mm. The e-grams at different focusing time were overlapped (labeled at the right).

Figure 7a. Dynamic Focusing of pI makers in pH 3-10 Servalyts

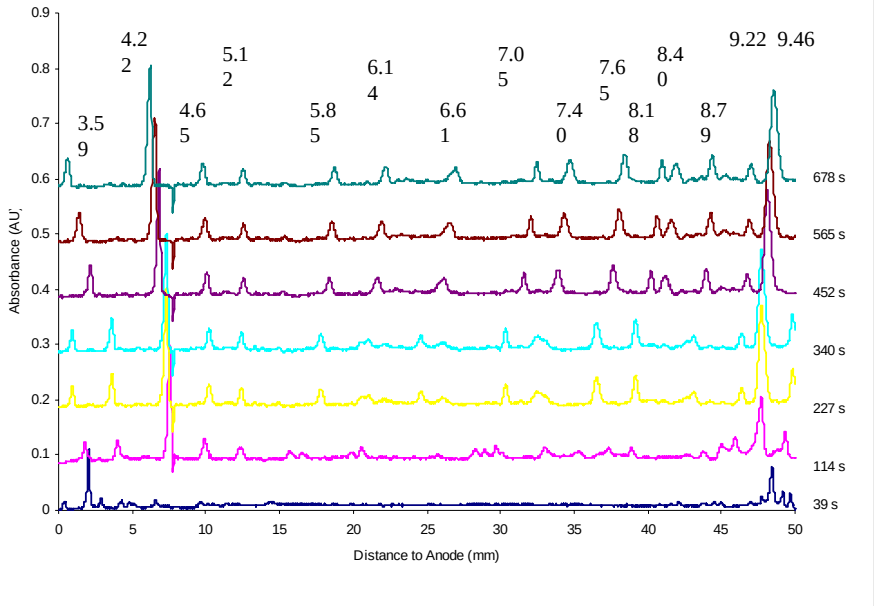
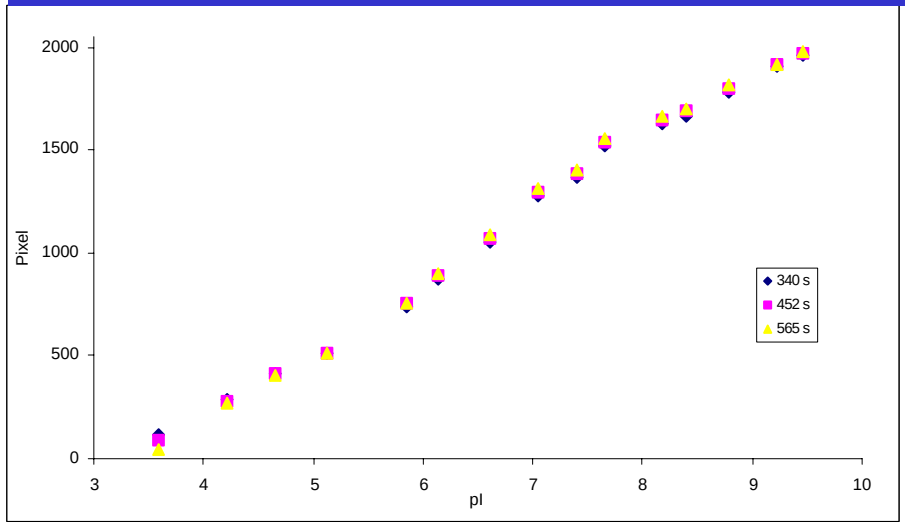


Figure 7b. Linearity of pH 3-10 Servalyts at Different Focusing Time



Experimental Conditions: pI markers were mixed with 8% (V/V) pH 3-10 Servalytes and 0.35% (W/V) methyl cellulose. Focusing voltage was 60V/mm. The e-grams at different focusing times were overlapped (labeled at the right).

Figure 8a. Dynamic Focusing of pI makers in 8 M Urea and pH 3-10 Servalyts

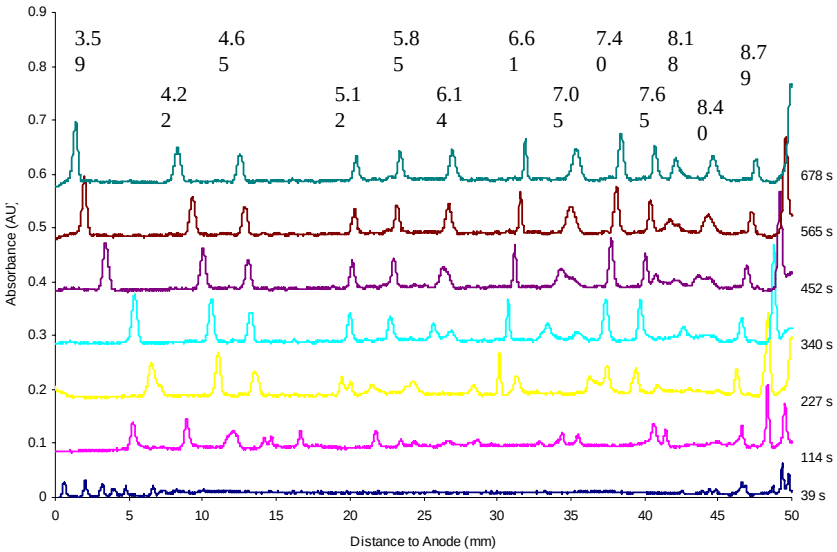
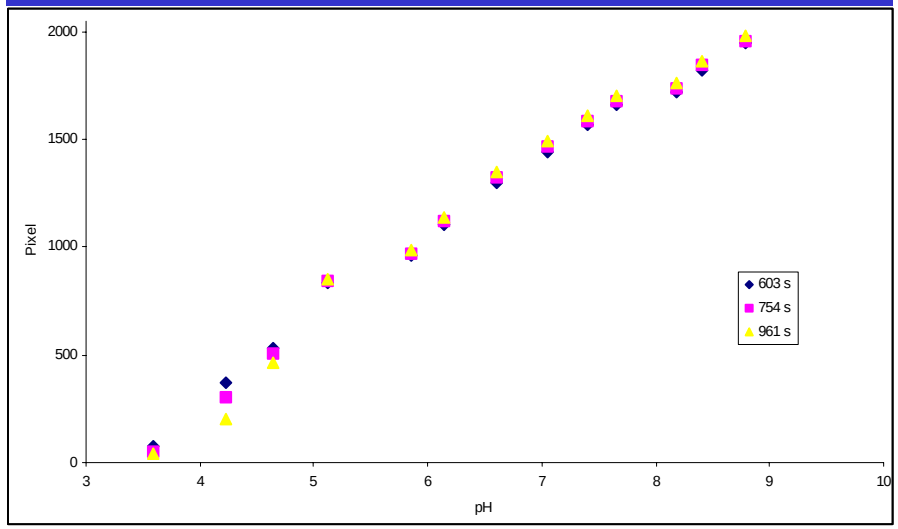


Figure 8b. Linearity of pH 3-10 Servalyts in 8 M Urea at Different Focusing Time



Experimental Conditions: pI markers were mixed with 8 M Urea, 8% (V/V) pH 3-10 Servalyts and 0.35% (W/V) methyl cellulose. Focusing voltage was 60V/mm. The e-grams at different focusing times were overlapped (labeled at the right).

Conclusions

1. It was observed that the focusing time and peak shapes of pI markers at different CAs were different.

2. Overall, the pH gradient was linear for the four CAs (r^2>0.99); however, the pH gradients of these CAs always had curvatures in some pH regions. The locations of the curvatures were unique for individual CAs.

3. Overall, the pH gradient was linear for all the four CAs in 8 M urea (r^2>0.90); however, it showed that the presence of 8 M urea significantly slowed down the pI marker focusing, narrowed the pH range of the CAs and changed the pH gradient in the acidic and basic regions for the four CAs compared to non-urea conditions.

4. The pH range observed experimentally with the iCE280 for specific CAs may be different from that provided by the respective vendors (pI markers 3.21 and 3.59 were lost in pH 3-10 Biolytes and pI markers 3.21 and 9.77 were lost in pH 3-10 Servalyts).

5. The experiment results showed that the stability of the pH gradient of the broad pH range Ampholine, Biolytes, Pharmalytes and Servalyts over time was good although it varied among CAs.

6. The pI marker shifting with focusing time was unique to individual CAs, which strongly suggests that this shifting was the result of a significant amount of non focusing components in synthetic CAs.

7. **Based on the results, in CIEF method development we recommend you:**
    1. **Select the proper focusing time.**
    2. **Select the pI marker pair with pIs close to that of the protein sample.**
    3. **Avoid selecting pI markers with pIs too close to the extreme of the specific pH range of the CAs.**
    4. **Avoid over focusing protein samples.**

References

1. Chrambach A. Anal. Biochem. 1972, 72,109-117.
2. Righetti P.G. J Chromatography A 2004, 1037, 491-499.
3. Wu J., Huang T. Electrophoresis 2006, 27, 3584- 3590.
4. Simó C, Citterio A., Righetti P.G. Electrophoresis 2007, 28, 3156-3162.

Materials

1. pI Markers - Convergent Bioscience, Toronto, ON, Canada
2. Servalyts® – Crescent Chemical, Islandia, NY
3. Biolytes® – Bio-Rad, Mississauga, ON, Canada
4. Pharmalytes®, Ampholine® – Sigma- Aldridge, Oakville, Canada