

# Chromatography

## Capillary Isoelectric Focusing – A Comparison of Different Carrier Ampholytes for Monoclonal Antibody Charge Heterogeneity Analysis

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This article will compare the performance of four commercially available brands of wide-range pH 3 to 10 carrier ampholytes (CA's), Pharmalyte, Servalyt, HR, and SH AESlyte, for high-resolution capillary isoelectric focusing on fluorocarbon coated capillaries. The carrier ampholytes-specific background was increased for Servalyt, and slightly increased for SH AESlyte in comparison to Pharmalyte and HR AESlyte. With all tested brands of carrier ampholytes, it was possible to analyse monoclonal antibody charge isoforms with high resolution and precision. The peak pattern of the test sample showing multiple isoform peaks in the pH range 6 to 7 was similar with Pharmalyte and HR AESlyte. A slightly increased resolution was observed with SH AESlyte, and the best resolution with Servalyt. The intermediate precision of experimentally determined isoelectric points obtained with three different capillary batches on three different days was better than 0.1 %RSD for all brands of carrier ampholytes and all isoforms. In terms of percent area, the observed intermediate precision was better than 3 %RSD for Pharmalyte and HR AESlyte, and better than 9 %RSD for SH AESlyte and Servalyt, with one exception.

### Introduction

Capillary electrophoresis (CE) is a well-suited analytical tool for protein characterisation due to its simple instrumentation, superior separation efficiency, small sample consumption, and short analysis time. Complementary information of the proteins is provided by different separation modes, including capillary zone electrophoresis (CZE), capillary isoelectric focusing (cIEF), and sodium dodecyl sulphate-capillary gel electrophoresis (SDS-CGE). A powerful method for protein charge heterogeneity analysis is cIEF. In this experimental setup, the proteins in the sample solution that initially fill up the whole capillary, are focused into sharp bands according to their isoelectric points in a pH gradient along the capillary [1]. The pH gradient is stabilised by carrier ampholytes. They are complex mixtures of small (200 – 1,200 Da) amphoteric molecules that are good carriers of conductivity and buffering capacity at their respective isoelectric point (pI) [2]. The choice of the CA brand is an important consideration for every cIEF experiment because the composition of CAs varies [2], and influences the separation performance. CAs with different chemical structures and ionisable groups have been introduced and marketed under trade names such as Pharmalyte, Servalyt and AESlyte. This study shows the impact of different commercially available carrier ampholyte brands on the performance of a high resolution cIEF method for charge heterogeneity analysis of monoclonal antibody (mAb) samples [3,4,5]. The fluorocarbon-coated capillaries used in this study proved to have a robust and reliable performance in cIEF combined with an exceptional longevity [6].

### Experimental

#### Materials

Methyl cellulose, urea, L-arginine, iminodiacetic acid and tris(hydroxymethyl)aminomethane (Tris) were obtained from Sigma Aldrich (St. Louis, MO, USA). Hydrochloric acid and glacial acetic acid were sourced from Merck Millipore (Darmstadt, Germany) and phosphoric acid from JT Baker (Austin, TX, USA). Pharmalyte 3-10 were from GE Healthcare (Freiburg, Germany), Servalyt 3-10 from Serva Electrophoresis (Heidelberg, Germany), HR AESlyte 3-10 and SH AESlyte 3-10 from Advanced Electrophoresis Solutions (Cambridge, Canada) and pI markers from Sciex (Framingham, MA, USA). A rat anti-DYKDDDDK mAb (part number 200474) and all other materials and instrumentation were obtained from Agilent Technologies (Waldbronn, Germany).

#### Sample preparation

Prior to CE analysis, the mAb test sample was desalted using Amicon Ultra 0.5 mL centrifugal filter devices (Merck Millipore, Darmstadt, Germany) and a buffer containing 20 mM Tris/HCl, pH 8. The protein concentration of the desalted mAb, 3.7 mg/mL, was measured with the Qubit assay (Life Technologies, Paisley, UK). Methyl cellulose containing solutions were prepared as described [7]. Sample solutions for cIEF analysis were prepared by adding the following reagents into 0.5 mL microcentrifuge vials:

- 100 µL of 0.6% MC containing 3 M urea
- 4 to 12 µL of CAs pH 3-10
- 10 µL of 500 mM L-arginine (cathodic stabiliser)
- 1 µL of 200 mM iminodiacetic acid (anodic stabiliser)

- 1 µL of each pI marker
- 5 µL of desalted mAb

Mixtures were vortexed for 10 s, centrifuged for about 1 minute and transferred into 100 µL CE sample vials. Sample solutions were kept in the autosampler carousel of the CE instrument at about 10°C and analysed within 24 h. UV/vis absorbance spectra of tenfold diluted CA stock solutions in water were recorded using a Nanodrop 1000 spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA) with a path length of 1 mm.

#### CE conditions

For all CE runs, an Agilent 7100 CE instruments equipped with an external water bath set to 6°C, a 280 nm high pass detector filter assembly and 4 bar external pressure were used. A µSIL-FC capillary with an inner diameter of 50 µm was cut at both ends at a distance of 8.5 cm and 24.5 cm from the detection window, equipped with a green alignment interface and fitted into the capillary cassette. Once a day and after cleaning, the capillaries were conditioned as follows:

- High pressure flush at 3.5 bar
- 350 mM acetic acid for 5 min
- Water for 2 min
- 0.5% MC for 5 min

Prior to every run, capillaries were conditioned as follows:

- High pressure flush at 3.5 bar
- 4.3 M urea solution for 3 min
- Water for 2 min

Samples were injected by applying 2 bar high pressure for 100 seconds, followed by a water dip of both inlet and outlet.

Focusing was done for 10-12 minutes at 25 kV with 200 mM phosphoric acid as anolyte and 300 mM NaOH as catholyte. For chemical mobilisation, the outlet vial was exchanged for 350 mM acetic acid and 30 kV was applied for 28 to 30 minutes. After each run, a high-pressure flush at 3.5 bar with water was done for 2 min.

After every 6 runs, the capillaries were cleaned by flushing them at 1 bar with 0.1 M NaOH for 2 minutes and with water for 30 minutes. Prior to storage, the capillaries were flushed at 1 bar with water for 20 minutes, with methanol for 5 minutes, then dried (5-minute flush from an empty vial). All flushes were done in forward direction (i.e. pressure was applied to the inlet vial). The capillary temperature was kept at 20°C. The detection wavelength was 280/20 nm, the reference wavelength 550/100 nm and the response time 2 seconds. For all reagents 2 ml glass vials were used. The fill volume was 1.6 ml, except for the waste vials which were empty. All reagent vials were exchanged after six runs. Electrodes were inspected daily for the accumulation of dirt in the upper funnel, and, if necessary, cleaned as described in the user manual.

#### Data processing

Apparent isoelectric points were calculated by linear regression analysis of pI marker versus migrations time in MS Excel. Only pI marker 5.5 and 7.0 data for were used for the calculation. Relative peak area values (in %) were calculated with time corrected areas. Intermediate precisions were calculated with the Analyse-it for Excel statistics software package (Analyse-it Software, Leeds, UK).

## Results and Discussion

### Adjustment of CA concentration and focusing conditions

This study tested the separation performance of cIEF with different brands of CAs, with a mAb sample having multiple charge isoforms with isoelectric points between 6 and 7. To ensure a fair comparison, the concentration of every CA brand was adjusted according to two criteria:

- migration of pI marker 5.5 and 7.0 within a time window of 25 to 33 minutes, and
- a 3.5 to 4 minute migration time difference between these two pI markers (data not shown).

Optimised concentrations were 4.7% v/v Pharmalyte and HR AESlyte (6  $\mu$ L stock solution added to the sample solution), 9.0% v/v SH AESlyte (12  $\mu$ L), and 1.3% w/v Servalyt (4  $\mu$ L; this CA brand was supplied as a 40% w/v stock solution). With these relatively high concentrations of Pharmalyte, HR and SH AESlyte the most acidic part of the pH gradient couldn't be observed, leading to a frequent loss of pI marker 4.1 (data not shown). If this part of the pH gradient is of interest, lower CA concentrations have to be employed. The focusing time used was 10 minutes, except for Servalyt, where the focusing time had to be increased to 12 minutes to get complete focusing of all pI markers (data not shown).

### Carrier ampholyte specific background

The CA specific background was monitored by cIEF runs with injection of pI markers only (Figure 1). The lowest background was observed with Pharmalyte and HR AESlyte, and a slightly enhanced background with SH AESlyte. A significantly enhanced background was seen with Servalyt. In line with this observation, diluted stock solutions of Servalyt showed, in comparison to the other CA brands, a higher absorbance at 280 nm (data not shown).

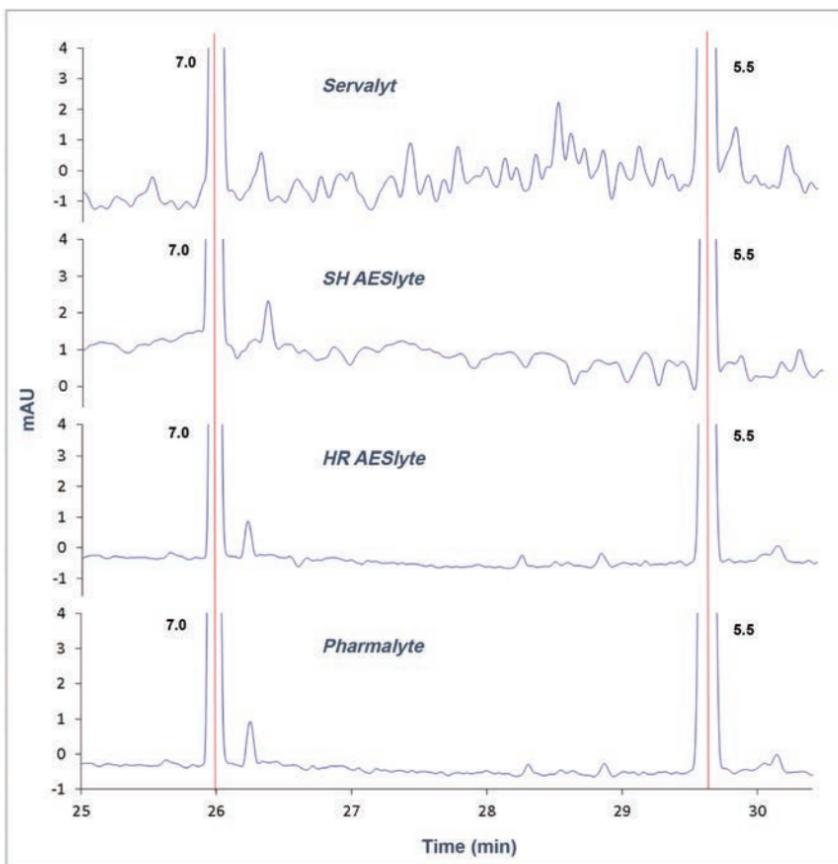


Figure 1. Background obtained with different CA brands. CIEF runs were done with sample solution without mAb test sample. Shown is a zoom in on the baseline the range between pI marker 7.0 and 5.5 of representative electropherograms obtained with Servalyt, SH AESlyte, HR AESlyte and Pharmalyte (from top to bottom). For better comparability, the time axis of the electropherograms were aligned with both pI markers as reference points (indicated by the red vertical lines).

### Resolution

Figure 2 shows electropherograms obtained for the mAb test sample with different CA brands. In comparison to the similar isoform pattern observed with Pharmalyte and HR AESlyte, the isoform pattern obtained with SH AESlyte and Servalyt was shifted by 0.10 to 0.15 pH units to the more acidic range. Seven isoforms could be clearly resolved, and automatically integrated with Pharmalyte and HR AESlyte. A slightly increased resolution was observed for SH AESlyte, with additional shoulders for peaks in the middle of the isoform pattern. However, a lower resolution was observed for the more acidic isoforms (SH AESlyte isoforms 6 and 7 in comparison to HR AESlyte and Pharmalyte isoforms 5 to 7, Figure 2). As for Pharmalyte and HR AESlyte, seven isoform peaks were automatically integrated with SH AESlyte (Figure 2). The highest resolution was obtained with Servalyt, that permitted identification of nine isoform peaks. This CA brand produced an overall somewhat different isoform pattern in comparison to the other three CA brands (Figure 2). The higher resolution obtained with Servalyt, in comparison to Pharmalyte, is in agreement with the higher content of CA isoforms in the pH range 6 to 8 [3] by exploring, through a 3-D methodology (Rotofor fractionation followed by CE MS).

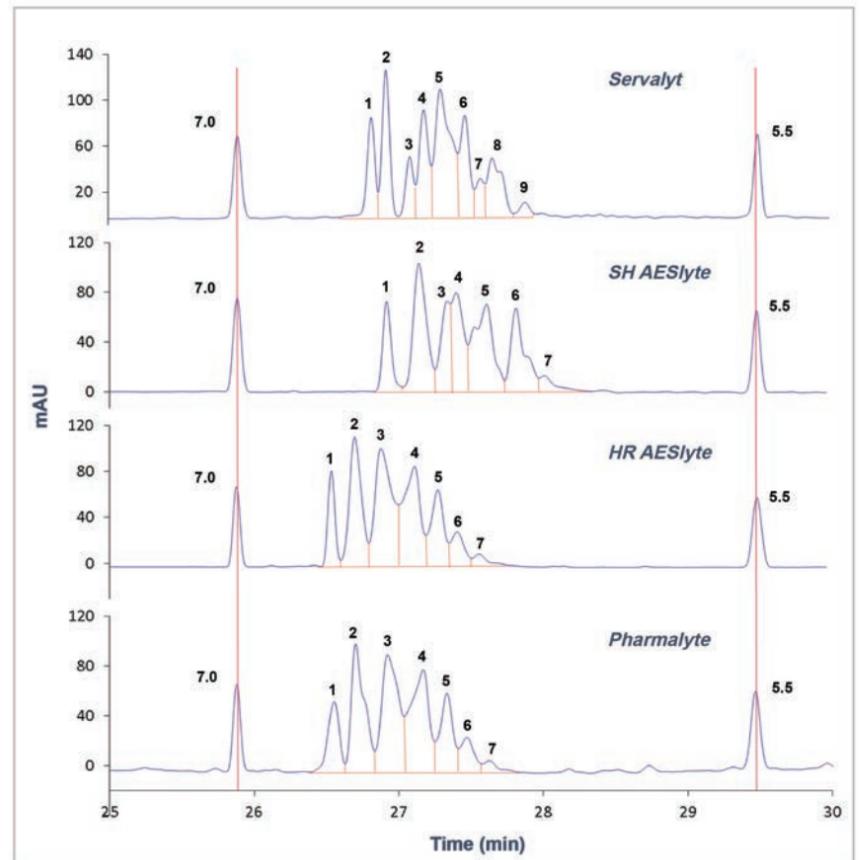


Figure 2. CIEF analysis of the mAb test sample with different CA brands. Shown is the range between pI marker 7.0 and 5.5 of representative electropherograms obtained with Servalyt, SH AESlyte, HR AESlyte and Pharmalyte (from top to bottom). For better comparability, the time axis of the electropherograms were aligned with both pI markers as reference points (indicated by the red vertical lines). Isoform peaks that were automatically integrated are consecutively numbered from basic to acidic. Integration limits are indicated by red dotted lines.

Table 1. Intermediate precision of apparent pI measured with different CA brands. For each CA brand sets of 6 runs were done on 3 different days and with 3 capillary batches (n=18). For the assignment of isoform peaks refer to Figure 2.

Isoform	Pharmalyte		HR AESlyte		SH AESlyte		Servalyt	
	Average	RSD%	Average	RSD%	Average	RSD%	Average	RSD%
1	6.720	0.033	6.727	0.006	6.570	0.026	6.615	0.011
2	6.655	0.029	6.664	0.065	6.477	0.036	6.572	0.013
3	6.564	0.011	6.583	0.005	6.394	0.030	6.503	0.005
4	6.461	0.022	6.487	0.015	6.369	0.025	6.463	0.019
5	6.392	0.019	6.419	0.011	6.282	0.029	6.416	0.018
6	6.335	0.023	6.363	0.014	6.198	0.028	6.344	0.010
7	6.269	0.020	6.300	0.013	6.114	0.022	6.301	0.020
8	na	na	na	na	na	na	6.265	0.006
9	na	na	na	na	na	na	6.171	0.012

### Intermediate Precision

The precision of the method including within-laboratory variations such as different day and capillary batch was determined for isoform pI and relative abundance of the mAb test sample (Tables 1 and 2). The intermediate precision for apparent pI obtained with all CA brands for all mAb isoforms was below 0.1 %RSD (Table 1). For relative peak area, the intermediate precision obtained with Pharmalyte and HR AESlyte was less than 3 %RSD for all isoforms (Table 2). With SH AESlyte and Servalyt, values less than 9 %RSD were observed. The only exception was with Servalyt, where the relatively small and poorly resolved isoform peak 7 showed an intermediate precision for relative peak area of 14 %RSD (Table 2 and Figure 2). The enhanced variability seen with SH AESlyte and Servalyt could be due to the more complex isoform pattern, making reliable integration more challenging (Figure 2), or the higher background observed with both CA brands (Figure 1). Overall, the precision results obtained with all tested CA brands agree well with published data obtained with a variety of different capillary coatings [4,6]. An interesting behaviour was observed with HR AESlyte for isoform peak 2, which showed a fourfold higher pI variability than any other peak with this CA brand (Table 1). In one set of measurements performed under repeatability conditions, this peak seems to switch between two shapes, one symmetrical and one with a small shoulder, as is observed with Pharmalyte (Figures 3 and 2), which might indicate some issues with sample stability.

Table 2. Intermediate precision of relative peak area (%) measured with different CA brands. For each CA brand sets of 6 runs were done on 3 different days and with 3 capillary batches (n=18). For the assignment of isoform peaks refer to Figure 2.

Isoform	Pharmalyte		HR AESlyte		SH AESlyte		Servalyt	
	Average	RSD%	Average	RSD%	Average	RSD%	Average	RSD%
1	10.3	1.2	9.1	0.9	10.6	3.5	10.6	0.8
2	20.7	0.6	20.8	0.5	21.5	3.0	14.4	1.4
3	25.0	0.8	26.1	1.9	11.5	6.7	6.1	2.4
4	23.0	0.7	22.4	2.9	14.3	3.0	12.9	3.9
5	12.4	0.7	12.7	1.3	22.7	3.2	24.4	5.0
6	5.8	0.9	5.9	1.0	16.0	3.5	14.0	8.7
7	2.8	2.9	2.9	2.6	3.5	7.2	3.9	14.3
8	na	na	na	na	na	na	11.3	2.7
9	na	na	na </td <td>na</td> <td>na</td> <td>na</td> <td>2.4</td> <td>1.2</td>	na	na	na	2.4	1.2

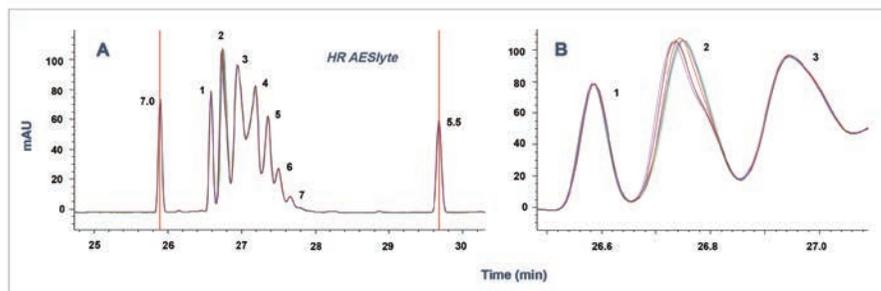


Figure 3. Overlay of 6 CIEF consecutive runs obtained with HR AESlyte on a single day with the same capillary. Shown is the range between pI marker 7.0 and 5.5 (A) and a zoom in on mAb isoforms 1-3 (B). For better comparability, the time axis of the electropherograms were aligned with both pI markers as reference points (indicated by the red vertical lines).

## Conclusion

This study shows that mAb charge heterogeneity analysis by high resolution cIEF on fluorocarbon-coated capillaries works well with different commercially available CA brands. All tested CA brands enabled the measurement of mAb charge isoform pI and relative peak area with high precision.

Performance differences between CA brands were observed in terms of resolution and CA-specific background. A similar performance was observed for Pharmalyte and HR AESlyte. In comparison to these CA brands, a slightly increased resolution and background was observed for SH AESlyte. The best resolution was observed for Servalyt.

However, this CA brand also showed the highest CA-specific background. Given the good performance with different CA brands combined with the high stability and ease-of-use of fluorocarbon-coated capillaries, the deployed high-resolution cIEF method presents an attractive choice for the characterisation of proteins in the biopharmaceutical industry.

## References

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