Using laser induced fluorescence to improve CE-SDS analyses

Maximize your sensitivity and resolution in capillary electrophoresis (CE-SDS) using modern chemistry for LIF detection

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As customers continue to develop more complicated protein therapeutics, there is an increasing demand for improvements in the resolution and sensitivity of platform capillary electrophoresis (CE) methods[1] traditionally used in the quality control of monoclonal antibodies. Laser induced fluorescence (LIF) has been used to improve the sensitivity of capillary gel electrophoresis with sodium dodecyl sulphate (CE-SDS), but often involves additional sample preparation or the use of hazardous chemicals, such as sodium cyanide required for FQ derivatization.[2] Recently, new chemistry has been used to profile adeno-associated viral (AAV) proteins[3] by CE-SDS. This new chemistry employed a simple two-step derivatized reaction using a pyrillium dye and eliminated the need for extensive sample preparation or the use of toxic chemicals. The method produced over a 100-fold increase in sensitivity over UV detection, and continued to use the same recommended standard CE-SDS separation method and gel as the UV method.

In this project we compare this new LIF-based methodology and the standard UV-based detection of a standard monoclonal antibody to determine if gains in resolution are possible using LIF.

We use the sensitivity gains from LIF derivatization together with applying CE theory to improve the resolution of the protein analysis, highlighting how LIF derivatization can provide resolution improvements in batch analysis. Figure 1 highlights how LIF can improve the resolution and sensitivity obtained for a standard antibody.

Key features of CE -SDS with LIF detection

- Removal of baseline artifacts improving peak integration
- Greater than a 100-fold improvement in sensitivity, making the method more versatile for new protein constructs that are at lower concentrations, such as proteins missing an FC region
- Lower sample consumption and injection amounts, reducing the requirements for additional wash steps between analyses
- In comparison to the standard UV method, similar resolution is obtained, and 40% faster separations are possible when using an optimized LIF method
- Higher resolutions are available if longer effective separation lengths are used

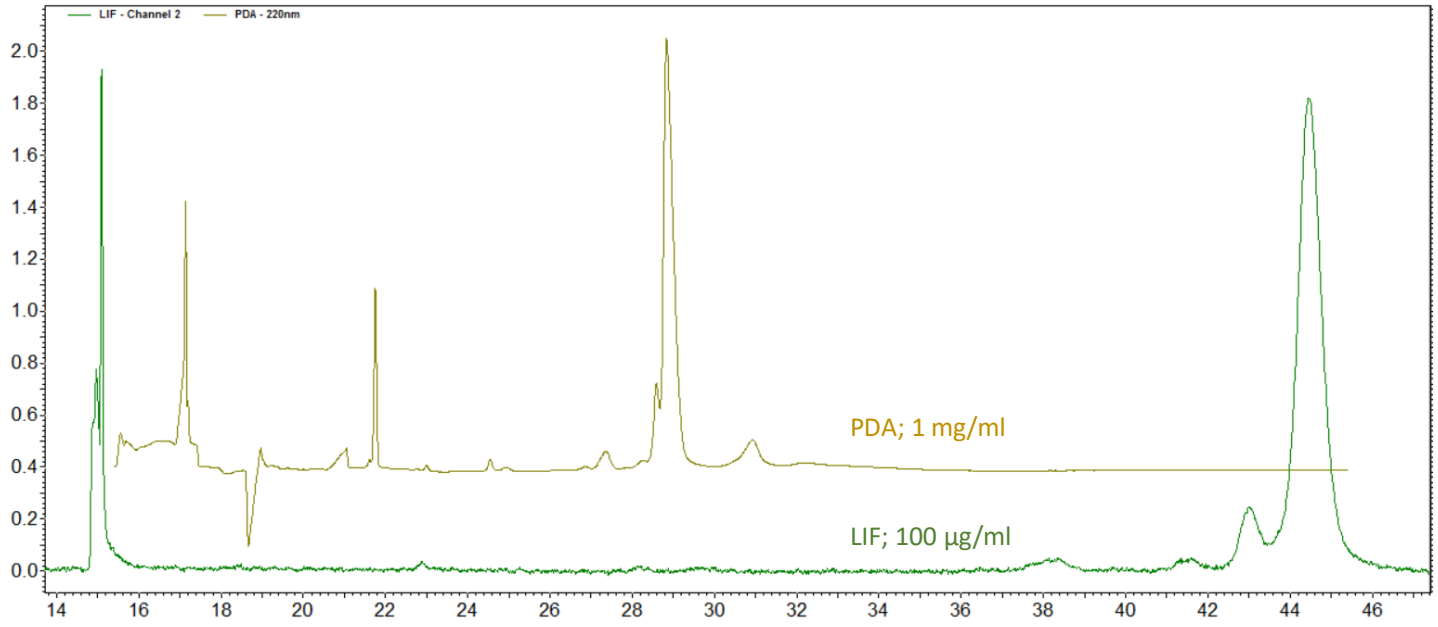

Figure 1. Overlaid electropherograms of the analysis of a monoclonal antibody using the standard CE-SDS analysis method with UV detection (5kv, 20s), and an optimized CE-SDS analysis using LIF detection (2.5 psi, 10 s injection).

Methods

Sample preparation: The IgG Control Standard and MW Size Standard were prepared as described in the commercial kit instructions. After heating the sample for 10 min at 70°C the proteins coated with SDS where ready for CE-SDS UV analysis.

For LIF analysis previously denatured protein sample was diluted with CE grade water (PN C44792). To the diluted sample, Chromeo dye solution[3] (5 µL, 1 mg/mL in methanol/water) was added and the mixture was heated (70 ºC, 10mins) before analysis.

For the development work, samples were diluted with CE grade water.

Capillary electrophoresis: Separations were performed with a prebuilt cartridge (part number A55625) on a PA 800 Plus Pharmaceutical Analysis System or using a 50.2 cm capillary (50 µm internal diameter) with an effective length of 40 cm. If not stated, standard separation methods (HR and HS) provided with the instrument where used. Depending on the experiment, capillary temperature, separation voltage, injection conditions and acquisition time where modified.

Detection: For CE-SDS-UV PDA detection was used at 220 nm. LIF detection used the solid-state laser available with the system at an excitation wavelength of 488 nm and emission filter wavelength of 600 nm (600 nm/80 nm band pass filter FWHM 12.5 mm; Edmond Optics Worldwide; PN 65736). The LIF detection system was equipped with the SCIEX LIF 2 Color Upgrade Kit (PN 144951) to avoid changing of filters between applications.

Data processing: Data was processed using the 32 Karat™ Software which comes as standard with the PA 800 Plus System.

Results and discussion

Figure 2 is the comparison of the same IgG sample analyzed by CE-SDS using UV detection with a PDA detector and one analyzed by LIF analysis after the protein had been derivatized. In this test, we analyzed the derivatized and underivatized sample using the same conditions, to directly compare the separations.

The LIF sample was diluted 10-fold and still produced a greater peak intensity compared to UV analysis. The baseline for the LIF analysis was also much closer to flat, with no peaks observed prior to the 10 kDa peak at 6 minutes. The peaks prior to the 10 kDa internal standard peak in the UV analysis were sample constituents that absorbed UV but were not proteinaceous in nature. In the LIF trace, the peak at 5 minutes was excess dye (low in this example as the sample was prepared by taking a 10 mg/mL standard, derivatizing and then diluting down to 0.1 mg/mL).

The protein profiles seen in UV and LIF were similar, but in the LIF detection the separation of the non-glycosylated species (NG, the minor peak at the front of the major protein IgG peak at 14 minutes) from the glycosylated species (IgG) was reduced. The pyrillium-based derivatization reagent reacted with the primary

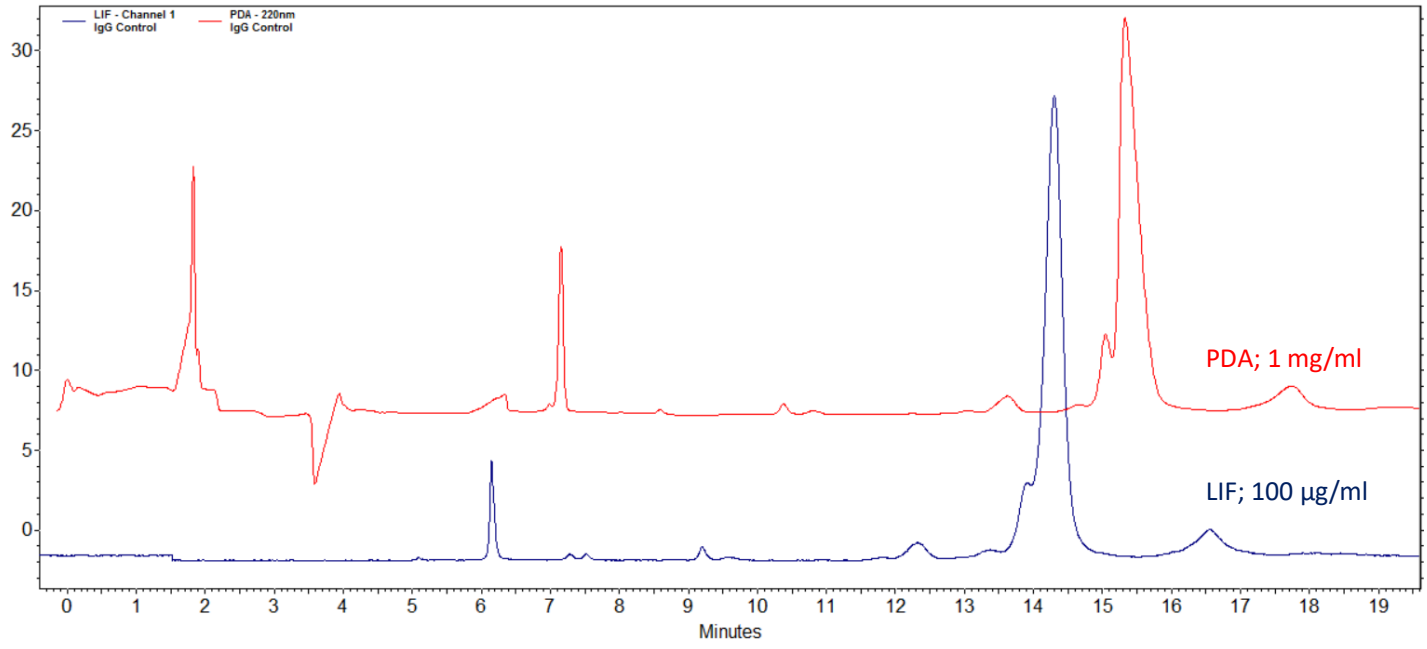

Figure 2. Red trace is the analysis of IgG (1 mg/mL) by CE-SDS using UV detection (PDA detector) offset by 1 minute. Blue trace is the analysis of IgG (0.1 mg/mL) by CE-SDS using LIF detection. In this example both samples were injected electrokinetically (5kV, 20 s)

amines found on the amino acid side chains and increased the size of the proteins, slightly reducing the resolution observed.

One of the effective ways to increase the resolution is to increase the temperature of the separation.[4] An example of this is shown in Figure 3. In Figure 2, the LIF separation used a higher temperature but a slightly lower voltage. The resolution obtained in this approach was higher than the PDA injection using the same effective length and the same capillary.

A classical way to improve resolution is to change the capillary length. This will increase the amount of time the sample is in the electric field. Figure 4 shows where the peaks broaden, but also how the resolution improves as the effective length increases.

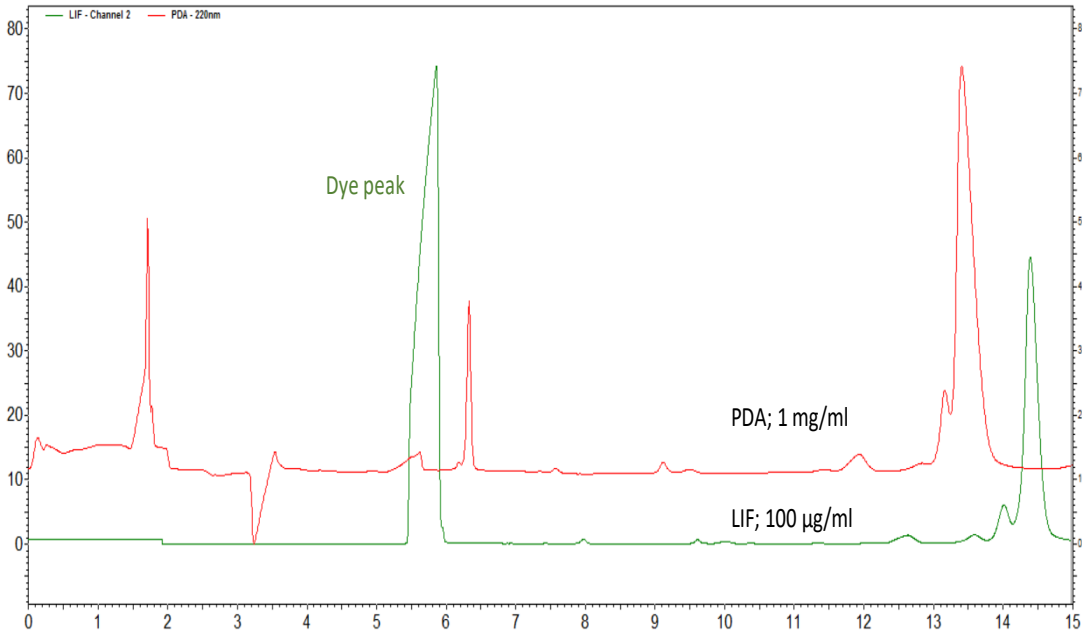

Figure 3. Comparison of an injection of an IgG using LIF detection (green trace, 13.5kV, 40 ºC) versus PDA detection (Red trace, 15kV, 25 ºC) using an effective separation length of 10 cm and the same injection conditions.

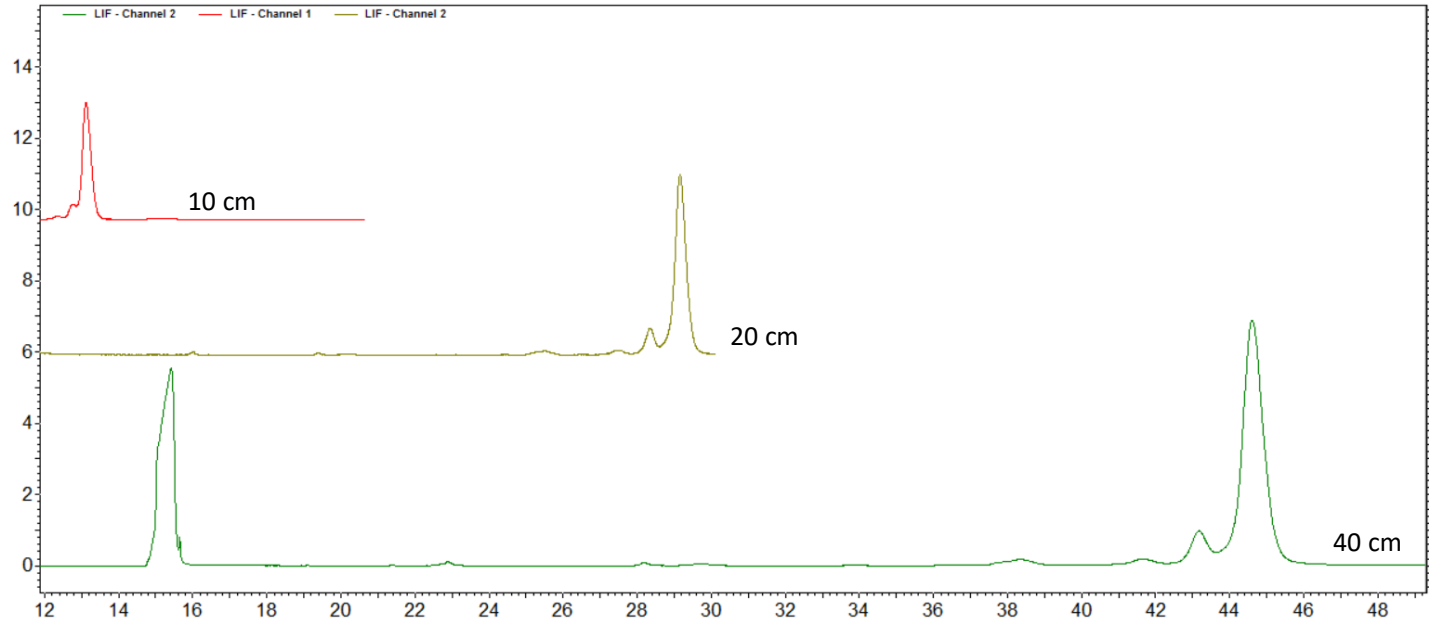

Figure 4. Comparison of an injection of 100 µg/mL IgG standard using LIF detection and different capillary lengths. Each sample was injected at 5kV, for 20 s at 45 ºC, with an effective field of 450V/cm.

The effect of capillary length and temperature on the separation of the NG species from the IgG parent peak is shown in Figure 5. By combining temperature with increasing effective separation length, and with only a marginal lower field strength, the best resolution is obtained.

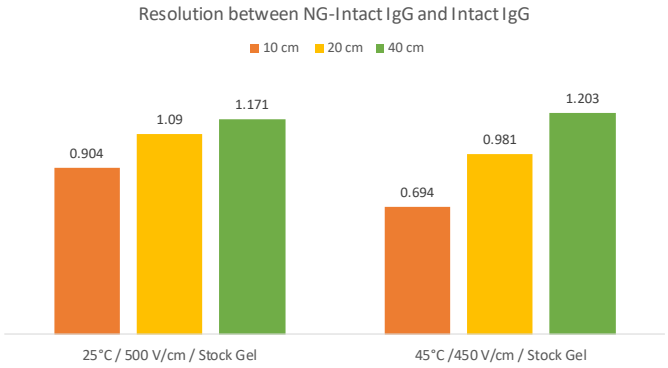

Figure 5. The impact of effective length and temperature on the separation and resolution of the NG species for the IgG parent peak using the same injection conditions.

The next way to improve resolution is to change the injection conditions. In the LIF method, sensitivity was not a challenge because the injection conditions already provided more than a 100-fold improvement in sensitivity.

Pressure injection was used to highlight the effect of injection conditions on the results, because it was easier to calculate the physical amount of protein loaded onto the capillary using pressure. Figure 6 highlights the effect on resolution of decreasing the amount injected.

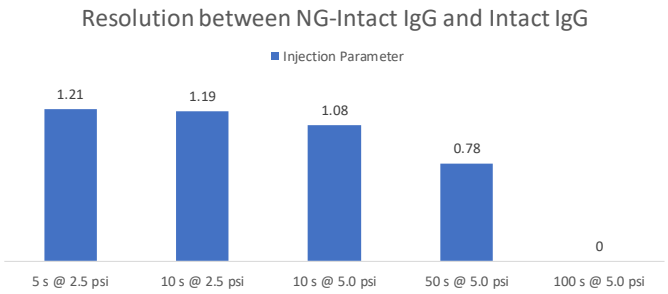

Figure 6. The effect of the injection amount on the resolution between NG and IgG peaks. All separations were performed at 13 kV, 40 ºC using an effective capillary length of 20 cm using a 100 µg/mL sample.

From this comparison we can observe that by injecting a smaller injection plug you improve resolution. This was also observed for electro kinetic injections and CZE separations[5] but also you get lower intensity peaks. The effect of sample concentration on resolution is shown in Figure 7. In Figure 7, we used the same injection conditions but different sample concentrations. As the sample concentration increased, the peak resolution remained the same, but the intensity increased.

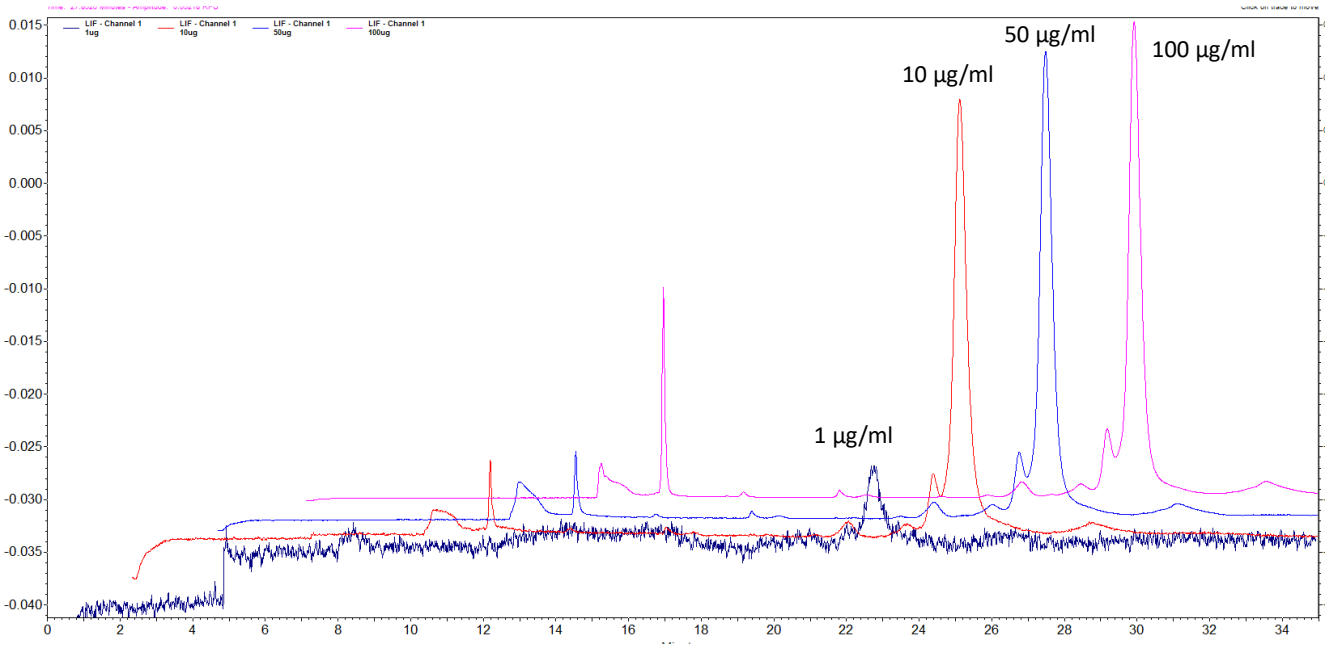

Figure 7. The effect of varying the sample concentration on peak resolution. Each sample (2.5 psi, 5 s injection) was analyzed using the same separation conditions (13 kV, effective separation length 20 cm, 40 ºC).

Taking all this into account, we compared a standard PDA analysis with the new LIF-based approach. In Figure 1, we showed that by using a longer effective separation length and an optimized method, you achieve the best resolution and nearly baseline separation of the NG and IgG parent peaks. In Figure 8, below, we compared a 10 cm effective separation length with an optimized LIF method to the standard UV method. In this comparison, you can get very similar resolution from the optimized LIF method, but faster separations compared to the classical UV method (20 cm effective separation length).

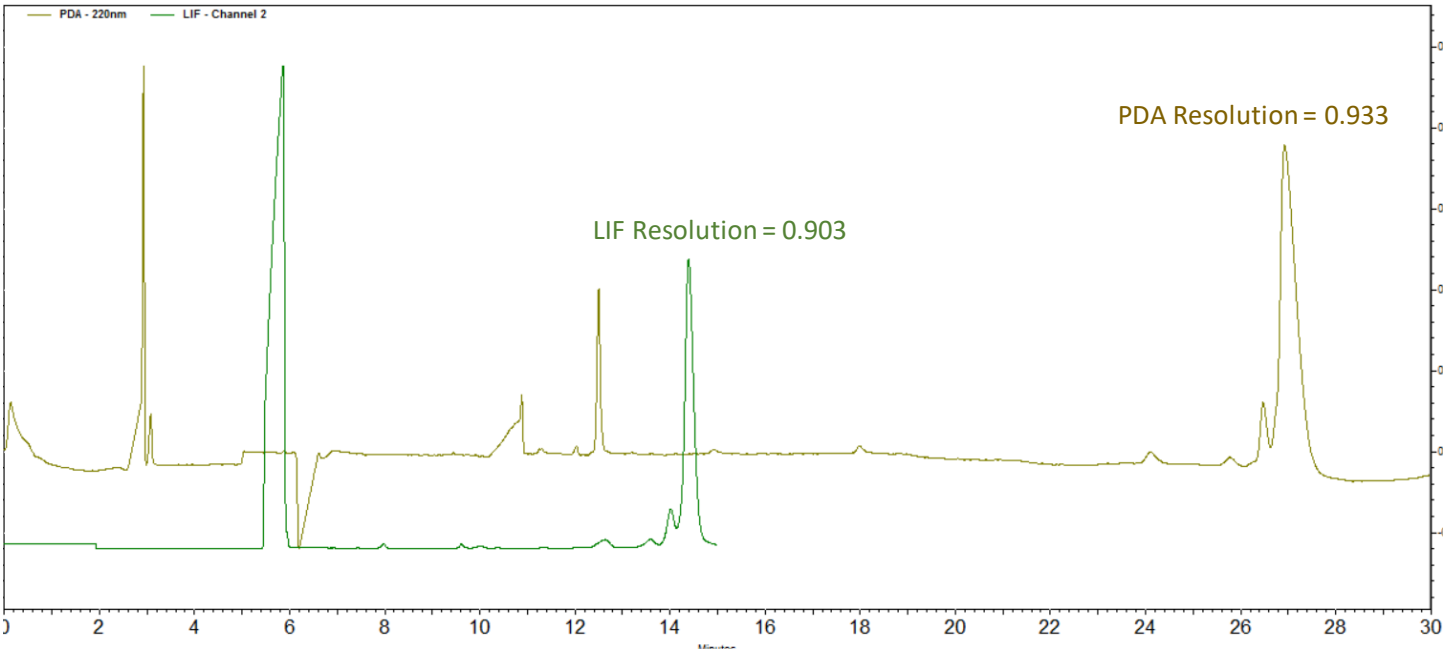

Figure 8. Comparison of optimized classical PDA UV method (20 cm effective separation length, brown trace) with optimized short LIF method (10 cm effective separation length green trace).

Conclusions

- LIF detection improved the sensitivity 100-1000-fold but also slightly reduces the resolution at the intact level under standard conditions.
- Changing the separation voltage and temperature improved the separation in LIF detection.
- Decreasing the injection plug size, but maintaining a higher concentration, improved the resolution for the LIF analysis. Combining this with an optimized separation temperature and voltage allowed LIF analyses to be faster on shorter capillaries while maintaining similar resolution to the standard PDA method.
- Using a longer capillary, and a similar effective field (higher separation voltage) provided the best resolution, outperforming the classic UV approach.

References

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