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Evaluation of custom injection programs and larger internal diameter capillary for strong solvent sample effects mitigation in liquid chromatography

Authors: Denis Andrei Ispan, Tibor Müllner, Maria Grübner

Themo Fisher Scientific, Germering, Germany

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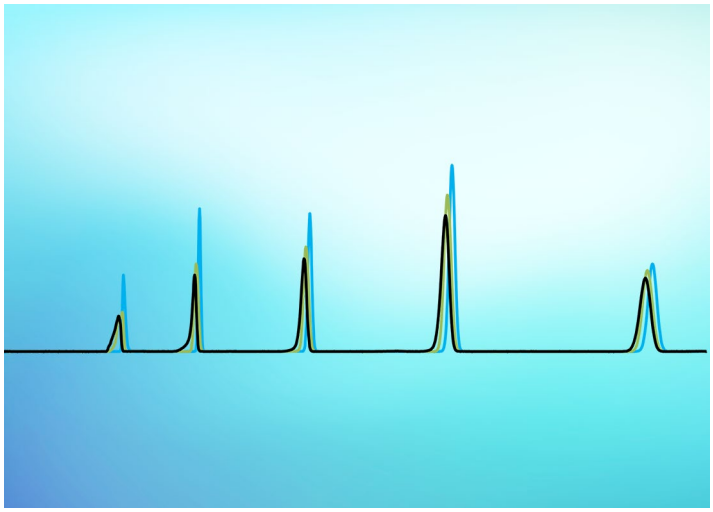

Goal

To determine the capability of custom injection programs and the strong solvent loop to reduce the strong solvent effects (SSE) caused by the sample on chromatographic performance for various diluents and column dimensions.

Introduction

Although in liquid chromatography (LC) analysis it is recommended to dissolve samples in weak eluting solvents,[1] occasionally samples must be injected in solvents stronger than initial mobile phase conditions (e.g., due to established analytical protocols, precipitation risks, the sample's solubility, detection limits, and many other factors). When the sample is dissolved in a solvent with a higher eluotropic strength/stronger elution power than the eluent, there is a risk of peak broadening, fronting, splitting or other peak distortions, which generally worsen with the diluent strength.[2] One of the common explanations for these peak distortions is the difference in eluotropic strength between the diluent and the mobile phase; another is a difference in viscosity causing hydrodynamic instability also known as "viscous fingering".[3] In most cases, it is difficult to distinguish between causes and therefore is referred to as the strong solvent effect (SSE), the sample diluent effect, or another similar term. In these instances, it is essential to find versatile strategies to deal with the possible consequences.

When the sample solvent is stronger than the eluent, the solvent plug must be thoroughly mixed with the mobile phase to obtain the expected retention of the analytes in the column. If the sample solvent is too strong and the mixing is not complete at the head of the column, some of the analytes or portions of them can (partially) break through the column with the strong diluent without

interacting with the stationary phase. This results in a loss of resolution and/or peak distortion causing irreproducible chromatographic performance. The wider the column diameter and the smaller the injection volume, the more mixing will be promoted and therefore the separation performance less affected.

Even if no strong solvent is involved, similar negative consequences may result from injection volumes that are too large for the current column characteristics. Beyond a certain level, the high analyte concentration can saturate the packing material and partially prevent further sample interactions with the stationary phase. In addition, the high volume of sample diluent, which adds dispersion, can result in peak broadening, peak distortion, and change in retention time depending on each specific scenario (column overload).

The SSE is also noteworthy when LC methods are transferred from older to modern LC instruments. The larger capillary and needle seat internal diameters (ID) in legacy instruments comprise a larger volume between the injector and the column and allow better mixing of mobile phase and sample. In addition, the column diameters used with such instruments are typically larger than with modern systems, and are usually kept when a method is transferred. With modern high-pressure liquid chromatography (HPLC) instruments, the SSE is intensified due to narrower capillaries and columns. As a result, corresponding mitigation strategies need to be employed in cases such as a compendial method transfer, which does not permit much flexibility for changes in sample composition, injection volume, or method setup. (According to USP General Chapter <621>,[4] the injection volume can be adjusted as long as the results are within the established precision, linearity, and detection limits.)

Several strategies have already been proposed to mitigate the SSE in LC systems without modifying the composition of the injected sample including at-column dilution by a weak eluent from a second pump,[5] inline mixing of the sample and diluent,[6] installation of a mixer or a large ID capillary between the injector and the column,[7] and application of custom injection programs (CIPs).[8] Inevitably, each strategy comes with advantages and disadvantages, and its suitability may depend on the specific application.

This technical note describes two of the mentioned strategies to overcome or reduce the SSE, namely the Strong Solvent Loop (SSL) and CIPs. Both approaches can deliver satisfying chromatographic results with limited effort or system adaptation. The SSE is shown and discussed at five injection levels, and the mitigation of SSE by implementing SSL and CIPs is evaluated for two column dimensions and three different sample solvents.

Experimental

Chemicals

- Deionized water, 18.2 MΩ·cm resistivity or higher
- Fisher Scientific™ Acetonitrile, Optima™ LC/MS grade (P/N A955-212)
- Uracil ≥99.0% (T), Sigma-Aldrich™ (P/N 94220)
- 4-Nitroaniline, 99%, ACROS Organics™ (P/N AC128371000)
- Methyl benzoate, 99%, ACROS Organics™ (P/N AC126340250)
- Phenetole, 98+%, ACROS Organics™ (P/N AC221491000)
- *o*-Xylene, 99%, pure, ACROS Organics™ (P/N AC140990010)

Sample handling

- Fisher Scientific™ Fisherbrand™ Mini Vortex Mixer (P/N 14-955-152)
- Thermo Scientific™ 11 mm Amber Glass Crimp/Snap Top Vials (P/N C4011-6W)
- Thermo Scientific™ 11 mm Autosampler Snap-It Caps (P/N C4011-54B)

Instrumentation

- Thermo Scientific™ Vanquish™ Core system consisting of:
  - System Base Vanquish Core (P/N VC-S01-A-02)
  - Vanquish Quaternary Pump C (P/N VC-P20-A)
  - Vanquish Split Sampler CT (P/N VC-A12-A)
  - Vanquish Thermostatted Column Compartment C (P/N VC-C10-A-03)
  - Vanquish Diode Array Detector CG (P/N VC-D11-A)
  - Standard flow cell, path length 10 mm (13 µL, SST, P/N 6083.0510)
  - Semi-micro flow cell, path length 7 mm (2.5 µL, SST P/N 6083.0530)
  - Strong solvent loop, V = 46.2 µL (P/N 6036.2200)

Sample preparation

The stock sample preparation was performed by mixing uracil at 1.5 mg/100 mL, *p*-nitroaniline 8 mg/100 mL, methyl benzoate 40 µL/100 mL, phenetole 150 µL/100 mL, and *o*-xylene 250 µL/100 mL in 50/50 ACN/H_2O (v/v). The resulting mixture is used for reversed-phase (RP) column performance tests including the ones employed in these analyses. To create samples in strong diluents, the mix was diluted (1/10) in acetonitrile (ACN), methanol (MeOH), dimethyl sulfoxide (DMSO), and in mobile phase (MP) as a reference point without the SSE.

Chromatographic conditions

Parameter	Value
Columns	Thermo Scientific™ Hypersil GOLD™, 150 × 3 mm, 3 µm (P/N 25003-153030) Thermo Scientific™ Hypersil GOLD™, 250 × 4.6 mm, 5 µm (P/N 25005-254630)
Mobile phase	A: Water 50% B: Acetonitrile 50%
Flow rate	0.425 mL/min with the 3 mm ID column 1.000 mL/min with the 4.6 mm ID column
Column temperature	30 °C
Autosampler temperature	6 °C
Injection volume	1, 5, 10, 20, and 30 µL
Detector settings	UV wavelength: 254 nm Data collection rate: 20 Hz Response time: 0 s
Flow cell	2.5 µL with the 3 mm ID column 13 µL with the 4.6 mm ID column

Table 2. CIP settings. "X" is a variable volume that depends on the sample injection volume. The water volume and the sample volume must sum to 100 µL. Other parameters like position offset, draw speed, and needle height can be configured in addition to the vial position and volume.

No.	Command	Parameters	Description
1	UDP_Prepare Liquid Handling	Volume=100 µL	Sets the total handling volume
2	UDP_Draw	Position=water vial, Volume=45 µL	Draws the first water plug of 45 µL from the specified vial position
3	UDP_Draw	*(Parameters not specified in CIP, so injection table properties are used)*	Draws the sample from the specified vial position and volume (1, 5, 10, 20, and 30 µL) in the injection table
4	UDP_Draw	Position=water vial, Volume=X µL	Draws the second water plug, which depends on the sample volume X = 100 µL – 45 µL sample volume
5	UDP_Wait	10 s	Move needle to injection port and wait 10 s before injection
6	UDP_ PrepareInject	-	End of liquid handling

Chromatography Data System

Thermo Scientific™ Chromeleon™ 7.3 Chromatography Data System (CDS) was used for data acquisition and analysis.

Results and discussion

The strong solvent effect

The SSE at two different column diameters over five injection volume levels is first evaluated in comparison to similar effects that may be caused by column overload. The extent of these effects is displayed for the samples dissolved in MP and in ACN.

Good practice in HPLC recommends that the maximum injectable volume is to be limited by the column length and cross-sectional area.[9] Nonetheless, the maximum injection volume still allowing suitable peak shapes may vary but also be dependent on the overall method conditions. If the injection volume is too high for the specific column, the peaks may be affected as a result of overload. The injection volume is also limited by the strength of the sample diluent in comparison to the MP: for stronger diluents the volume should be decreased, whereas for weaker diluents it can be increased.

Figure 1 illustrates the change of the resulting chromatograms for a 3 mm ID column as the injection volume is increased. When the sample is diluted in MP, the peaks slightly tail with increasing injection volume, likely due to column overload. However, when the sample is in strong diluent, the first (non-retained peak) is fronting and distorted at 5 µL and above because the mixing in front of the column is not sufficient, resulting in a solvent-mismatch effect. The retained peaks start fronting at 10 µL, more severely at 20 µL, and even result in shoulders with fronting at 30 µL. As the volume of injection is increased, less adequate mixing of the strong solvent with the MP takes place, carrying some of the analytes along without allowing sufficient interaction with the stationary phase.

For the 4.6 mm ID column (Figure 2), the sample diluted in MP results in optimal peak shapes. However, for the sample dissolved in strong diluent, the unretained peak shows fronting from 5 µL upwards and is less distorted. The other peaks start fronting only from injection levels of 20 µL and above, but to a much lesser extent than what has been observed with the 3 mm ID column.

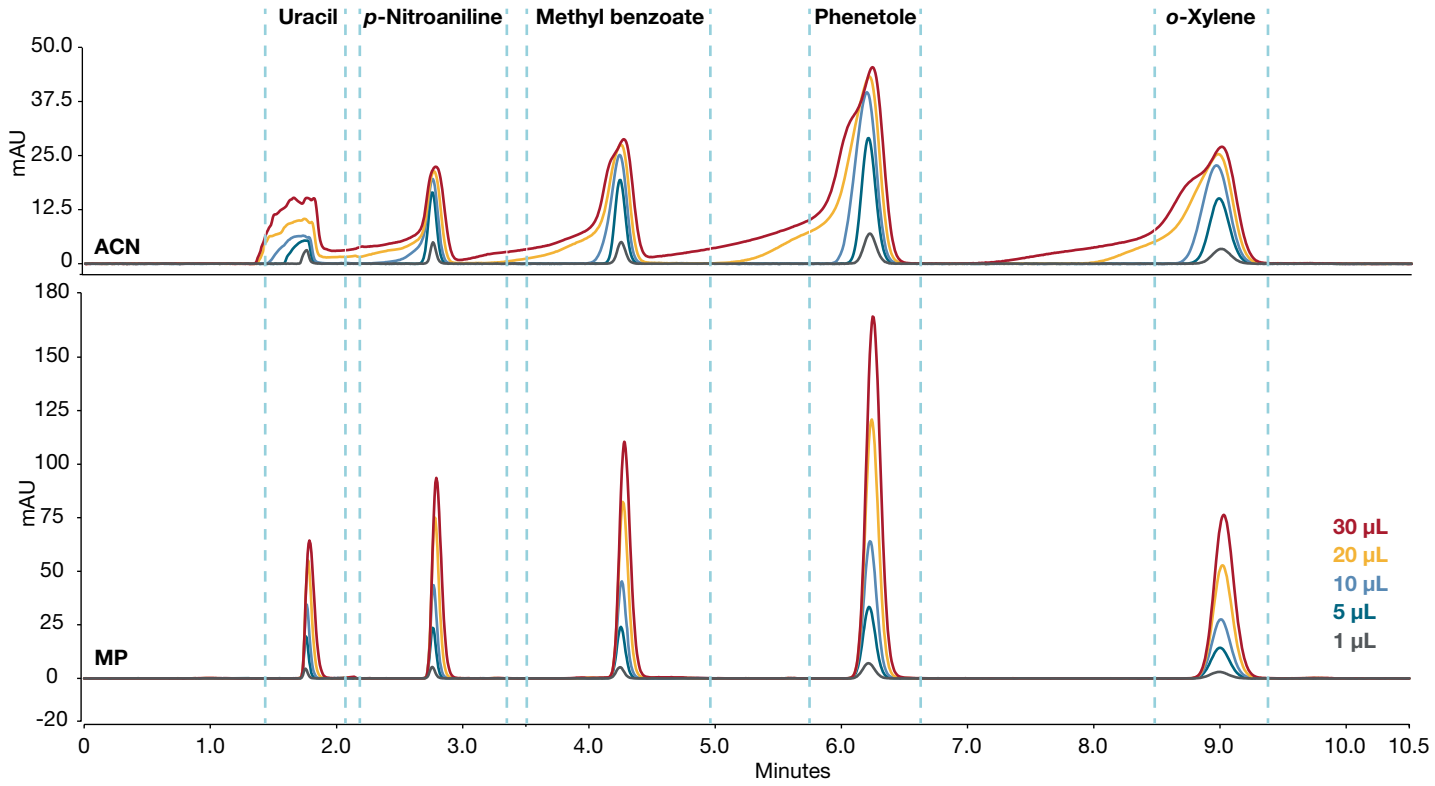

Figure 1. Overlay of chromatograms at different injection volumes (1, 5, 10, 20, and 30 µL) for the 3 mm ID column. Top: sample is dissolved in ACN; bottom: sample is dissolved in MP (50/50 ACN/water).

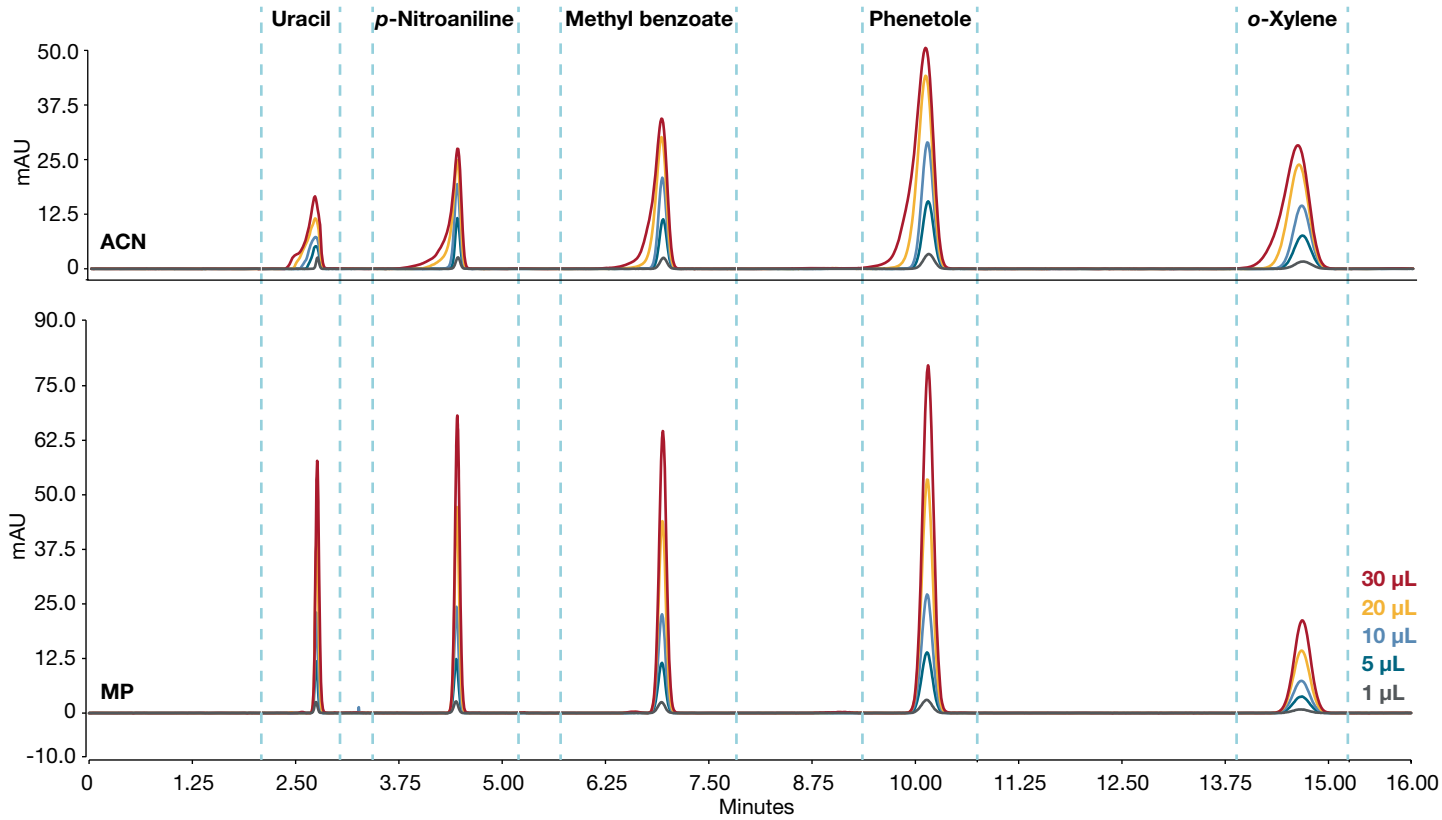

Figure 2. Overlay of chromatograms at different injection volumes (1, 5, 10, 20, and 30 µL) for the 4.6 mm I.D. column. Top: sample is dissolved in ACN; bottom: sample is dissolved in MP (50/50 ACN/water).

In the previous examples, the effect of a strong solvent injection was shown to be less significant with increasing column dimensions. Moreover, the extent of peak distortion depends on the retention time (or retention factor) of the analyte, with a substantial difference between a non-retained and a later eluting analyte. The difference between a column overload effect and the SSE for the current conditions was also observed. The first resulted in tailing and the second in fronting, shouldering, and peak distortion. In short, for both columns the unretained peak was always affected from the 5 µL injection upwards and the rest of the peaks were affected to a different degree, which also depends on the column size.

Figure 3. Schematic of the strong solvent loop on the left and installed on the instrument on the right

The SSE induced by MeOH and DMSO results in similar distortion to ACN, but at different intensities (data not shown). ACN and MeOH have more similar and stronger effects than DMSO. Also, under the given conditions, DMSO results in a considerable artifact pattern that interferes with the first and sometimes the second peak in the chromatogram.

Mitigation strategies by SSL and CIPs

Custom injection programs based on simple commands called UDPs (user-defined programs), provide the user the ability to individually control the injection process. Here, it is implemented on the Vanquish Core HPLC system for the reduction of the sample solvent strength by diluting the sample with a weaker diluent in the injection loop prior to injection.[8] For this study, the program is described in Table 2. The total injection volume was 100 µL and the loop was filled as follows: 45 µL of water, followed by the sample volume (1, 5, 10, 20, or 30 µL), and the remaining volume of water to yield 100 µL total.

The second strategy involves the Strong Solvent Loop (Figure 3), which is a large ID capillary to improve the mixing of the sample plug and the mobile phase. It is installed between the sampler valve and the column inlet capillary and is also an easy way to mimic the dispersion of old systems. Both SSL and CIP were shown to significantly improve the performance of a compendial method.[10] However, the SSL may limit the applicability of UHPLC methods due to increased dispersion. The use of the SSL implies a change in the fluidic configuration, meaning that system re-qualification may be required to fulfill the guidelines of regulated laboratories.

Therefore, to align with regulatory guidelines, all the implications should be considered for each specific case. Either SSE mitigation strategy aims to improve mixing before the sample reaches the column. The CIP by mixing the sample with lower strength diluent in the sample loop and the SSL by facilitating mixing of the sample plug with the mobile phase by increased dispersion.

Evaluation of the mitigation extent

In Figure 4, the mitigation of SSE using CIP is shown for the 3 mm ID column. An injection volume of 10 µL was selected as representative for such a column format. SSL was also tested for this injection volume and column diameter, with modest improvements. The lower effectiveness of the SSL under these conditions is due to the extra-column dispersion contribution outweighing the beneficial effects of improved mixing (data not shown). Figure 13 shows decrease of efficiency under current conditions when the SSL was installed. In comparison with the no mitigation chromatograms, the CIP clearly provides better chromatographic results with narrower and higher peaks. Good peak shape was even obtained up to 20 µL injection volume and up to 30 µL for the last two peaks with MeOH and the last three with DMSO (data not shown). In the current chromatograms the unretained peak is distorted in all three solvents. The second peak is affected differently overall, presenting poor peak shape. Nonetheless, with the CIP the chromatogram is considerably improved, and very good peak shapes are obtained in each case except for the unretained peak.

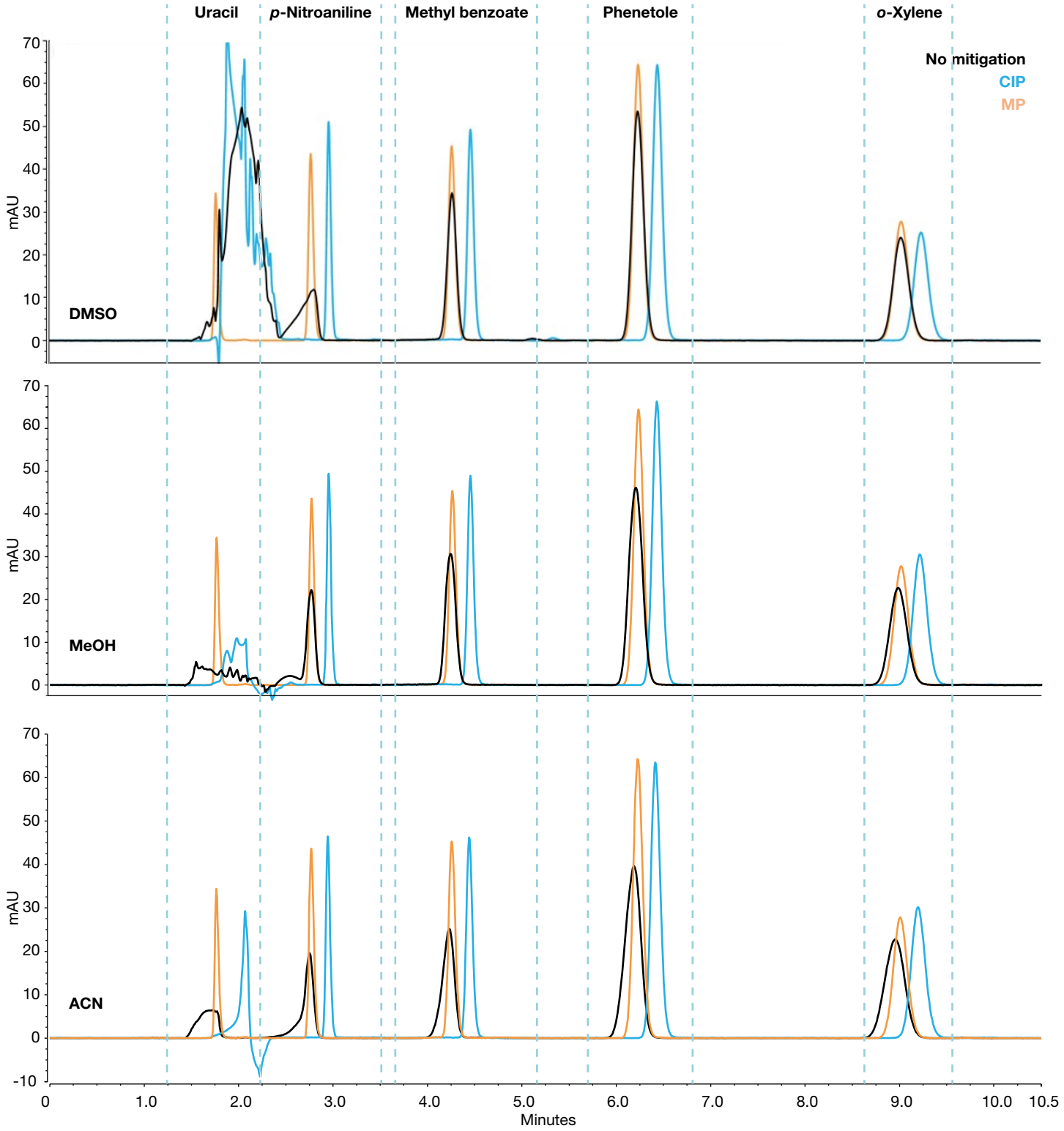

Figure 4. Overlay of chromatograms using the 3 mm ID column and 10 µL injection without any mitigation ("no mitigation" and sample dissolved in mobile phase (MP)) and with CIP for ACN, MeOH, and DMSO. The sample in MP is shown as a reference.

An increase in the retention time (RT) when using CIP is observed compared to the results without mitigation. This outcome is due to the added water volume in the injection loop that is in front of the sample in the order of elution. For the current injection of 10 µL, another 45 µL of water was added before and after to sum to the total 100 µL as explained in Table 2. Therefore, the sample plug reaches the column head later than usual.

The peak width, asymmetry, and relative retention time (RRT) were compared across different conditions to assess the impact of the SSE. Uracil and *p*-nitroaniline were excluded from the comparison because they could not be integrated at any condition, due to poor peak shape.

The peak width results of the three late eluting peaks using the 3 mm ID column are displayed in Figure 5. The first thing to notice is that although the width varies considerably between the solvents, the CIP provides very similar peak widths in all conditions. This indicates that for this column and solvents CIP efficacy is not affected by the sample solvent. As a result, the peak width with CIP is comparable to the ideal situation where the sample is dissolved in the mobile phase. When no mitigation is applied, the severity of SSE is consistent with solvent strength. Peaks without mitigation are broader with ACN, followed by MeOH and DMSO.

In Figure 6, the asymmetry values with CIP are comparable to the values without mitigation and solvent dissolved in MP. Often, even under conditions where SSE is strong, peak symmetry is satisfactory even without CIP mitigation. Therefore, the beneficial effects of CIP are not always as obvious as for the peak width.

RRT values in Figure 7 are consistent across all conditions, meaning that there is no negative and disproportionate effect of the peak RT shift due to the CIP.

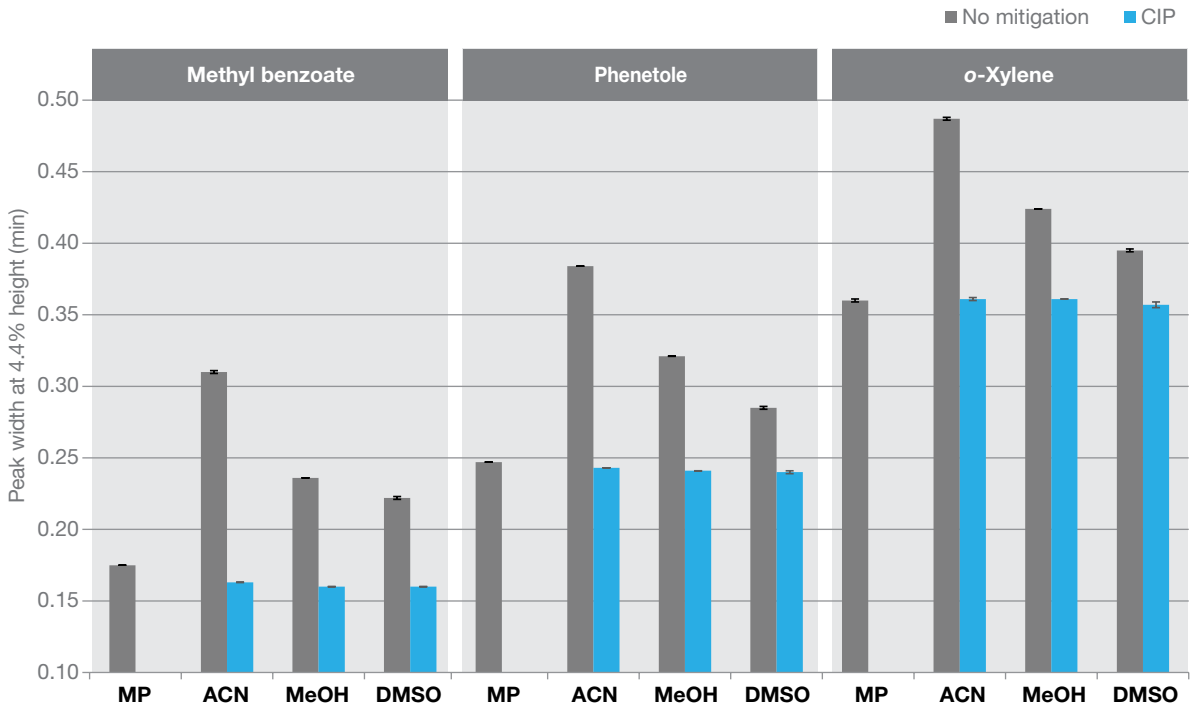

Figure 5. Peak width at 4.4% with 3 mm ID column and 10 µL injection volume with no mitigation and CIP for all sample solvents. Standard deviation is shown with the vertical error bars (N=3).

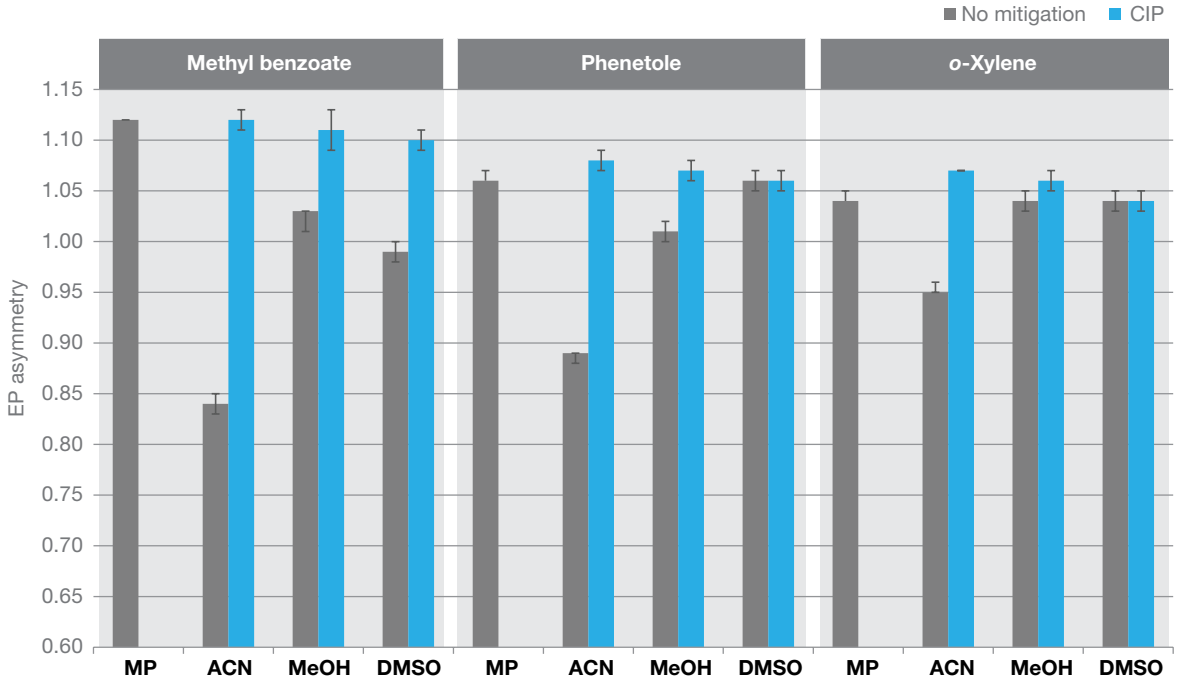

Figure 6. Peak asymmetry with 3 mm ID column and 10 µL injection volume with no mitigation and CIP for all sample solvents. Standard deviation is shown with the vertical error bars (N=3).

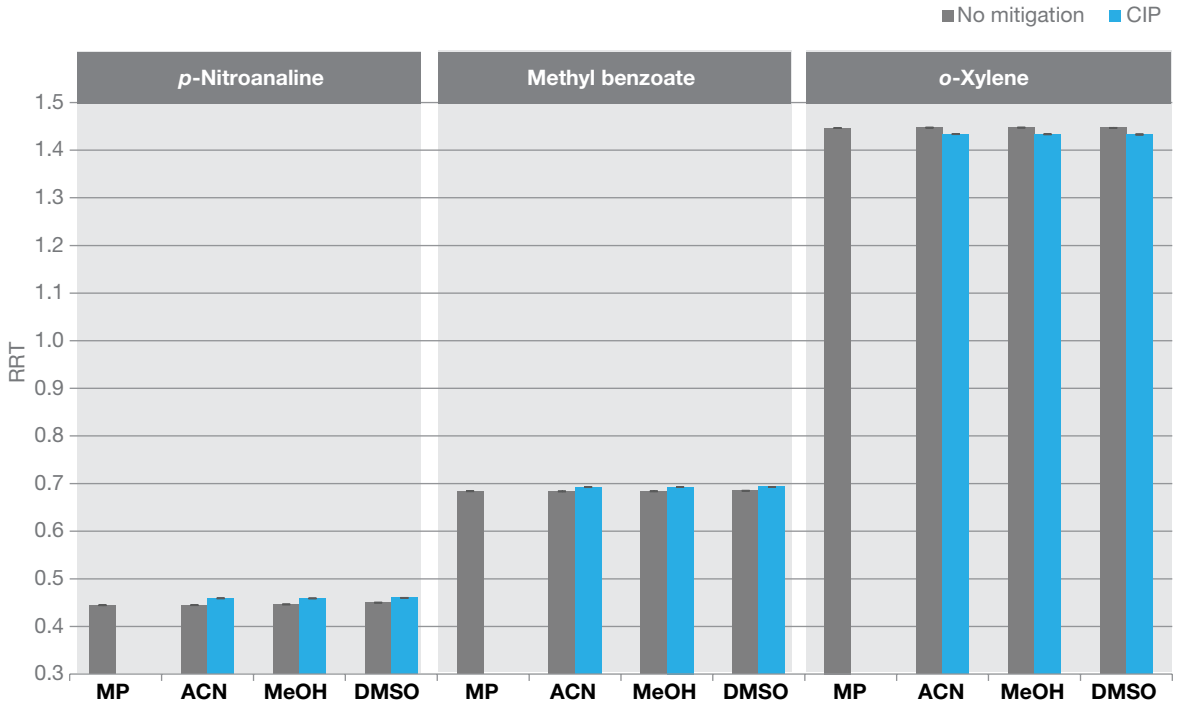

Figure 7. Retention time relative to phenetole (RRT) with 3 mm ID column and 10 µL injection volume with no mitigation and CIP for all sample solvents. Standard deviation is shown with the vertical error bars (N=3).

The chromatograms obtained with the 4.6 mm column ID with both mitigation techniques (CIP and SSL) and without are displayed in Figure 8 for 20 µL injection volume, a typical injection volume for this column format. The unretained uracil is again not detected for the MeOH and DMSO due to the baseline artifacts, but is detected as a sharp peak for the sample dissolved in ACN when CIP is implemented. For the peak shape of *p*-nitroaniline, which is more strongly affected by SSE than the later eluting peaks, the CIP brings improvement for all conditions. Moreover, in the case that the strong solvent is ACN, the SSL is also effective and satisfactory peak shape is obtained.

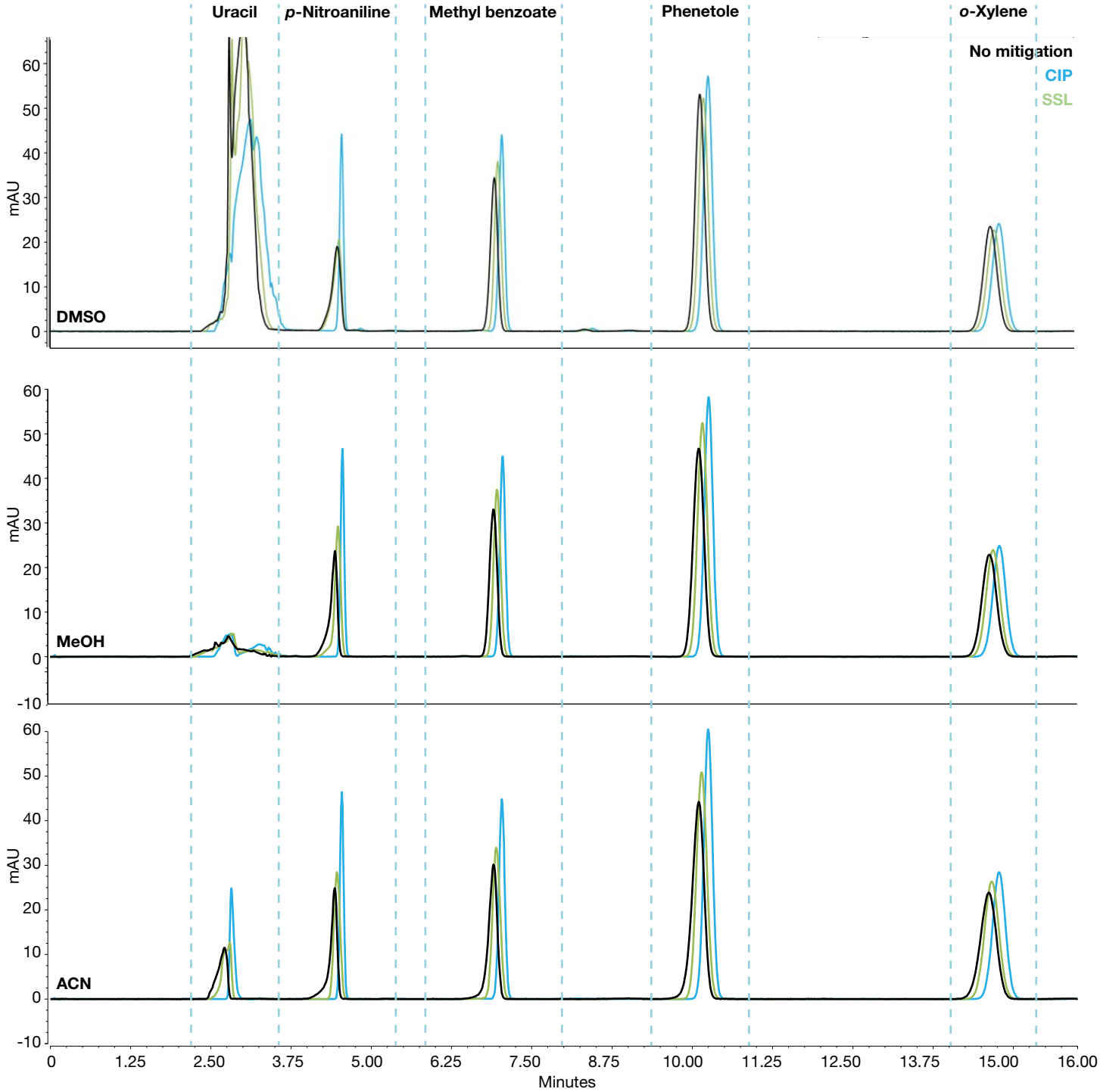

Figure 8. Overlay of chromatograms using the 4.6 mm ID column and 20 µL injection volume without any mitigation, with CIP and with SSL for all three sample solvents (ACN, MeOH, and DMSO)

The same behavior of peak width as for the 3 mm ID column (both for no mitigation and CIP) is observed for the 4.6 mm ID column (Figure 9). However, the SSL reduces the peak width more for ACN, to a lesser extent for MeOH, and even less for DMSO. Therefore, the SSL mitigation in comparison to the CIP is less effective and more dependent on the sample solvent strength.

The CIP and "no mitigation" asymmetry patterns are similar for the 4.6 mm ID column and the 3 mm one, but even more symmetric for the 4.6 mm (Figure 10). The SSL usually performs very well and comparable to the CIP delivering good peak symmetry.

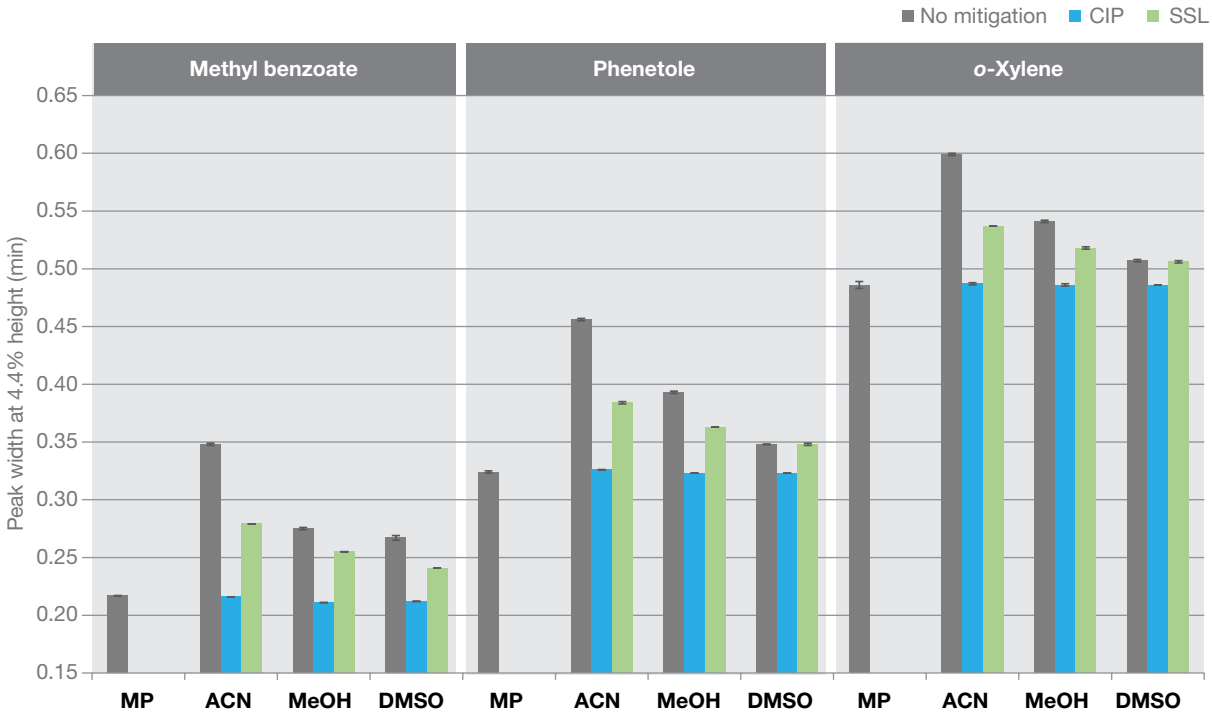

Figure 9. Peak width at 4.4% with the 4.6 mm ID column and 20 µL injection volume with no mitigation, CIP and SSL for all sample solvents. Standard deviation is shown with the vertical error bars (N=3).

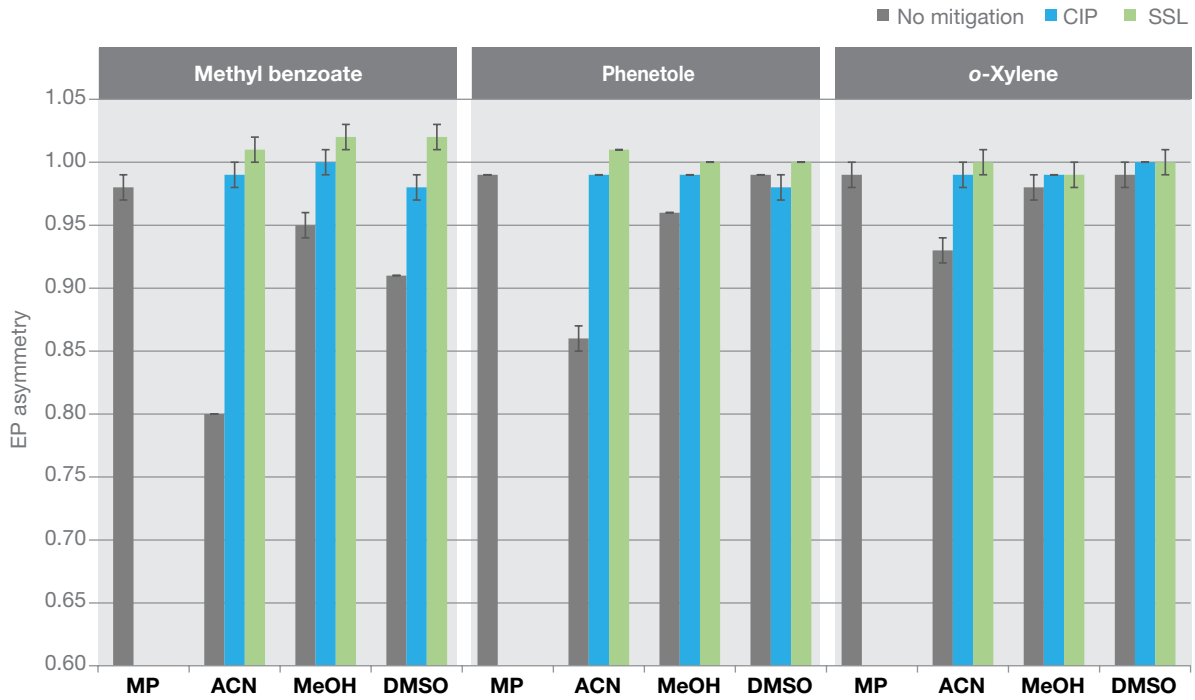

Figure 10. Peak asymmetry with the 4.6 mm ID column and 20 µL injection volume with no mitigation, CIP and SSL for all sample solvents. Standard deviation is shown with the vertical error bars (N=3).

The relative retention time (relative to phenetole) shows again that although different absolute RT with the SSL and CIP are obtained compared to the method without mitigation, it is identical for all three solvents and methods (Figure 11).

There may be more specific or uncommon cases where one of the mitigation strategies may not be enough to obtain appropriate results. For the use of CIP at high sample volumes, when the portion of weak diluent in the injection loop is relatively low compared to the sample solvent, it may happen that the SSE is not adequately mitigated because of insufficient mixing between weak and strong solvent. In that case, employing CIP together with the SSL to enable a better dilution of the strong solvent could improve the peak shape. Figure 12 shows chromatograms for SSL, CIP, and a combination of both using a sample volume of 30 µL for the 3 mm ID column.

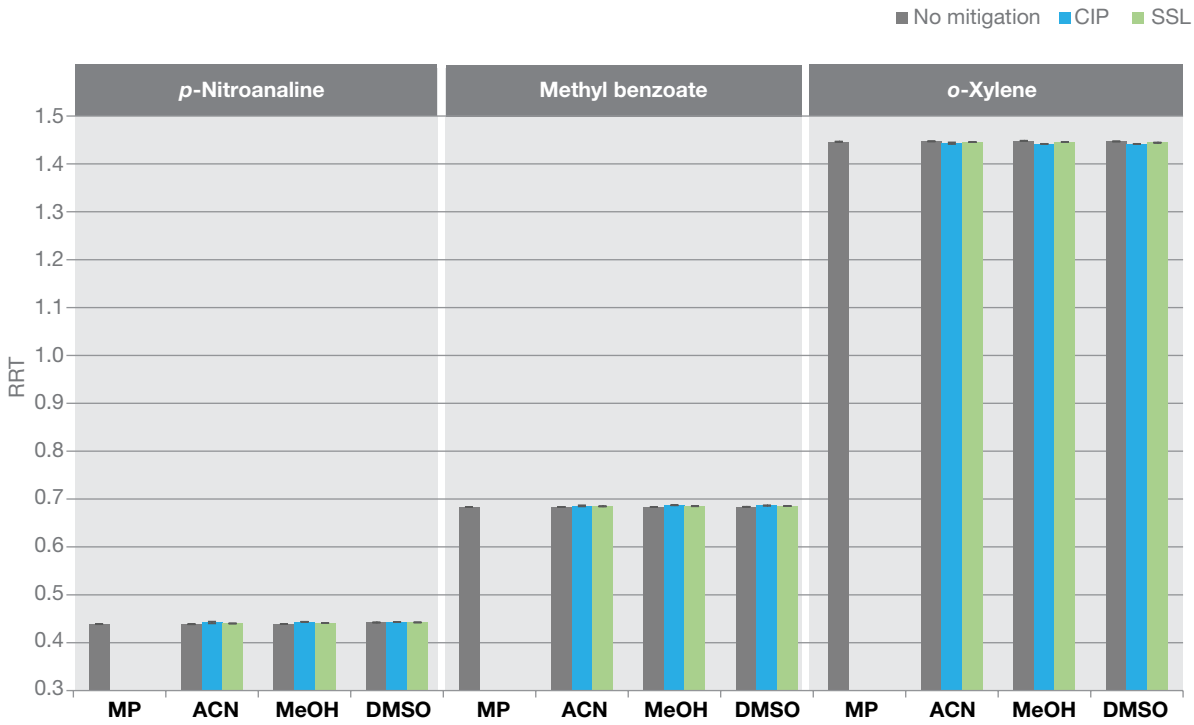

Figure 11. Retention time relative to phenetole (RRT) with the 4.6 mm ID column and 20 µL injection volume with no mitigation, CIP and SSL for all sample solvents. Standard deviation is shown with the vertical error bars (N=3).

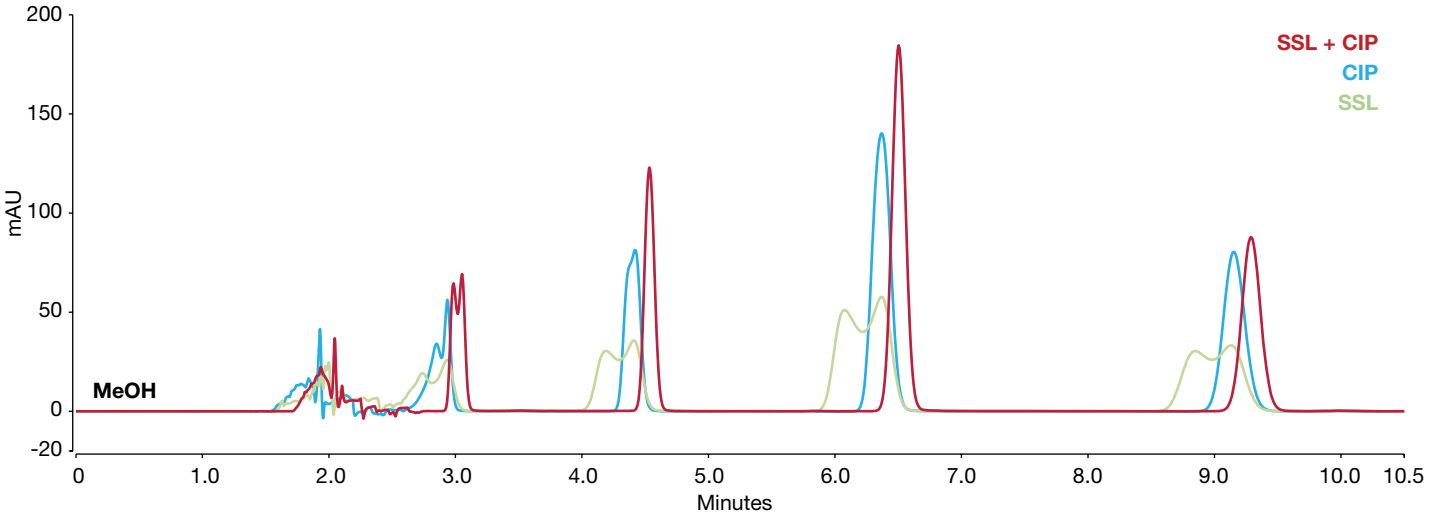

Figure 12. Overlay of chromatograms with SSL, CIP, and SSL+CIP using MeOH as a strong solvent for column 3 mm I.D and 30 µL injection

With methanol as the sample diluent, the first peak cannot be detected. The second peak is improved but still split even if both strategies are applied in combination. The third peak has a shoulder with CIP, but with CIP and SSL it becomes narrower and higher and the shoulder disappears. The behavior of the fourth peak is the same as the third peak. In contrast, if the sample is already well diluted by CIP, and the SSL is employed in addition, the extra dispersion may negatively affect the peak width.

Finally, a comparison of the efficiency between "no mitigation" and SSL when the sample is in normal conditions (MP) and no SSE is present was carried out (Figure 13). This setup may be important to consider if another method with no SSE is run on the same instrument. Therefore, to avoid requalification of the system, some loss in the efficiency might be a worth trade off. Clearly, due to the added dispersion when using the SSL, the efficiency will decrease. As noted, it is more considerable as the column has smaller ID and less as the RT increases.

It should be taken into consideration that the SSE as well as its mitigation potential effectiveness may strongly vary from method to method. Therefore, the presented results need to be considered as a relevant, but still limited, subset of typical conditions in reversed-phase chromatography. The extent of the related effects depends on many factors, such as if the method is gradient or isocratic, the sample solvent (diluent) and its elution strength, the injection volume, the retention factor of the respective analyte, the column dimensions, and stationary phase characteristics. Refer to the already cited application notes[8,10] to evaluate their use in other methods.

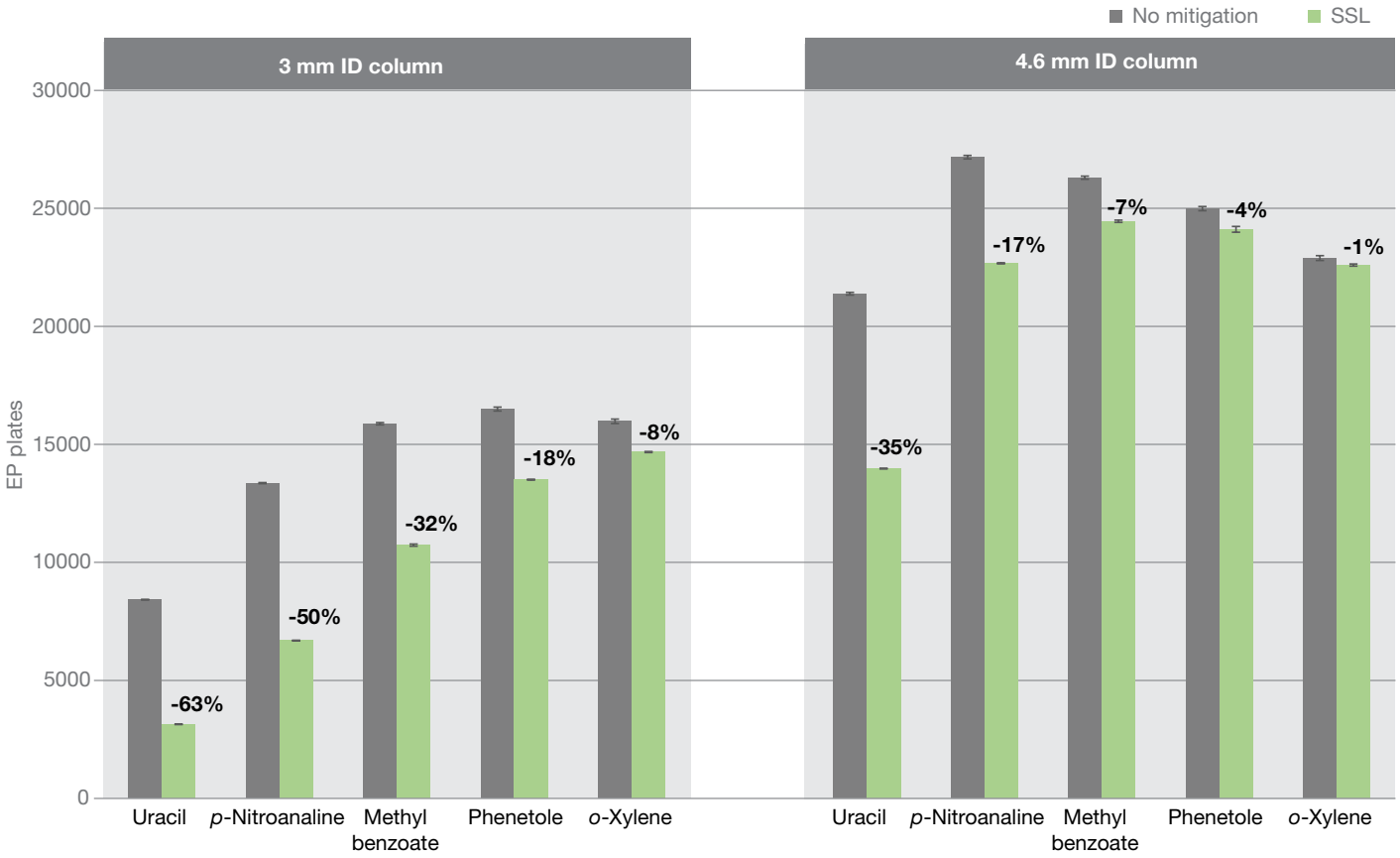

Figure 13. Comparison of the theoretical plates (N) of each peak between "no mitigation" and SSL when no SSE is present (sample in MP). For the 3 mm ID column, data of a 10 µL injection is shown and for the 4.6 mm ID column, of a 20 µL injection. The percentage refers to the decrease of plates from no mitigation to when SSL is installed. Standard deviation is shown with the vertical error bars (N=3).

The SSE, causing peak fronting, broadening, splitting, and distortion, is more relevant and intense as the dimensions of the column decrease and the injection volume increases. There is also a difference between the solvents that are used for the sample in the present conditions, with ACN typically having a more intense effect upon the analyzed peaks, then MeOH, and finally DMSO. Due to the mismatch of the MP and diluent and/or the detectability of the impurities in the diluent, the unretained peak was covered by artifacts in most cases. It may have also been caused by the viscous fingering effect, which was beyond the scope of this study to investigate. This may be a problem depending on the method and the retention of the peak of interest because the CIP or SSL may not be able to avoid it. Usually, peaks of interest have higher retention factors and therefore this would not be an issue. Nevertheless, the CIP provided satisfactory results in every case for the second peak (less retained and closer to time zero) and even for the unretained one for the 4.6 mm ID column with ACN.

It can be concluded that the CIP is an excellent mitigation strategy of the SSE for all the considered scenarios. In comparison with the SSL it is expected to give better results because weak solvent is used, which in this case is more polar and enables focusing the analytes at the column inlet (reverse effect than the strong solvent), while the SSL only provides mixing with the mobile phase. Further optimization regarding the injection volume and diluent in the injection loop can be made depending on the scope and the conditions of the method. In addition, CIP mitigation strength was not solvent dependent in the considered peaks for the peak width and only slightly for the asymmetry. For asymmetry, more variability was found overall, presenting better results than no mitigation for the 4.6 mm ID column but not for the 3 mm where in some occasions the peaks with strong solvent were less asymmetric.

The SSL generally has lower mitigation power when compared to the CIP in the current conditions. In addition, its mitigation effect depends more on the type of sample solvent and its miscibility with the MP. For instance, it can be more clearly observed in the peak width and its capacity to fix the second peak. For the analyzed peaks, the asymmetry does not depend as much on the solvent because the values are in the same range. Nonetheless, it was shown to reduce the SSE, improving the peak width and asymmetry in various circumstances and even eliminated the fronting in the second peak for ACN, which has a small retention factor. This approach may work well when the SSE is not very strong because of the pre-requisite that the sample needs to be mixed with the MP. It also can improve the peak shape further when used in addition to CIP. Lastly, if other methods have to be run on the same instrument where no mitigation is required, the tradeoff between requalification (if needed)/loss of efficiency and SSE mitigation should be evaluated.

Conclusion

- The SSE intensifies as the dimensions of the column decrease and the injection volume increases, affecting more severely the unretained peak and distorting the rest of the peaks with shoulders, broadening, fronting, and splitting.
- The CIP is an excellent mitigation strategy of the SSE, which improves peak shape by narrowing it in the majority of the circumstances.
- The SSL improves the peaks in most of the scenarios although not as much as the CIP, and in some occasions, it can provide good peak shapes when the CIP is not sufficient by adding it in the configuration.
- The SSL is much more intuitive to use than programming a CIP. Nonetheless, the CIP is relatively easy to set up once the parameters are understood and permits many variations and setups.

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