Technical Overview

The Agilent InfinityLab 2D-LC Solution with Active Solvent Modulation

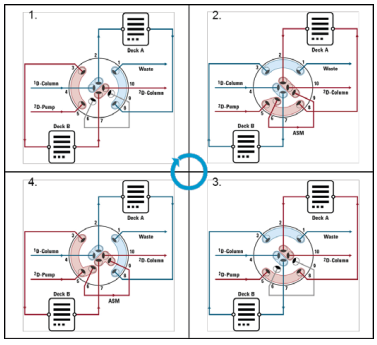
Achieving Improved Resolution and Sensitivity for Challenging Combinations of Separation Conditions

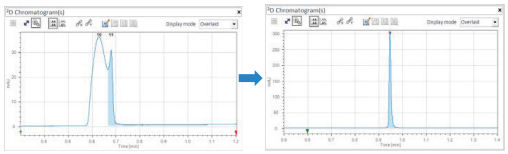
Author

Sonja Krieger
Agilent Technologies, Inc.

Abstract

A major challenge during method development in two-dimensional liquid chromatography (2D-LC) can be the incompatibility between the first (^{1}D) and second dimension (^{2}D) solvent systems. For challenging combinations of separation systems, volume overload effects such as breakthrough or broad and distorted peaks in the ^{2}D can be observed. Active solvent modulation (ASM) provides a valve-based approach to address this problem, leading to improved resolution and sensitivity in the ^{2}D. This Technical Overview explains the principle of ASM, and demonstrates its effect in the combination of two reversed-phase (RP) LC separations and a combination of hydrophilic interaction liquid chromatography (HILIC) and RPLC.





Introduction

In situations where the sample to be analyzed is complex, or contains compounds of interest that are difficult to separate, the separation that can be achieved using one-dimensional liquid chromatography (1D-LC) might not be sufficient. Two-dimensional liquid chromatography (2D-LC) is then the method of choice[1]. The Agilent InfinityLab 2D-LC Solution enables comprehensive (LCxLC), multiple heart-cutting (MHC), and high-resolution sampling (HiRes) 2D-LC to be performed. Therefore, enabling either improved separation for the entire sample (LCxLC) or separation of target compounds (MHC and HiRes 2D-LC), that could not be separated using 1D-LC.

One challenge in 2D-LC method development can be the incompatibility between the first (^{1}D) and second dimension (^{2}D) solvent systems[2]. In loop-based 2D-LC, the analyte is transferred from the ^{1}D to the ^{2}D in the ^{1}D effluent. When the volume of ^{1}D effluent that is transferred to the ^{2}D becomes significant relative to the ^{2}D column dead volume, the ^{1}D effluent essentially becomes the ^{2}D mobile phase for a certain amount of time[1]. For combinations of separation systems where the analytes show low retention on the ^{2}D column in the ^{1}D effluent, this can lead to volume overload effects, such as breakthrough or broad and distorted peaks in the ^{2}D[1,2]. Active solvent modulation (ASM) addresses this problem by diluting the ^{1}D effluent with ^{2}D mobile phase ahead of the ^{2}D column, enabling focusing of the analytes at the ^{2}D column head[2].

This Technical Overview explains the principle of active solvent modulation, and demonstrates its effect in the combination of two reversed-phase (RP) LC separations as well as a combination of hydrophilic interaction liquid chromatography (HILIC) and RPLC.

Experimental

Equipment

The Agilent 1290 Infinity II 2D-LC System comprised the following modules:

- Two Agilent 1290 Infinity II High Speed Pumps (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167B) with cooler (option #100)
- Two Agilent 1290 Infinity II Multicolumn Thermostats (G7116B)
- Two Agilent 1290 Infinity II Diode Array Detectors (G7117B) with a 10-mm Max-Light Cartridge Cell (G4212-60008)
- Agilent 1290 Infinity Valve Drive (G1170A) with 2D-LC Valve, Active Solvent Modulation (G4243A)
- Two Agilent 1290 Infinity Valve Drives (G1170A) with Multiple heart-cutting Valves (G4242-64000) equipped with 40-µL loops

Mass selective detection (MSD) was performed using an Agilent 6150 Single Quadrupole LC/MS (G6150BA) equipped with an Agilent Jet Stream ESI source (G1958-65538).

Software

Agilent OpenLAB CDS ChemStation Edition Rev. C.01.07 SR3 [465] with 2D-LC Software version A.01.04.

Columns

- Agilent ZORBAX Eclipse Plus PAH, 2.1 × 100 mm, 1.8 µm (p/n 959764-918)
- Agilent ZORBAX Eclipse Plus C8 RRHD, 2.1 × 50 mm, 1.8 µm (p/n 959757-906)
- Agilent ZORBAX HILIC Plus, 2.1 × 100 mm, 3.5 µm (p/n 959793-901)
- Agilent AdvanceBio Peptide Mapping, 2.1 × 100 mm, 2.7 µm (p/n 655750-902)

Chemicals

All solvents were LC grade. Acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22-µm membrane point-of-use cartridge (Millipak, EMD Millipore, Billerica, MA, USA). Ammonium formate and formic acid were purchased from Sigma-Aldrich (Steinheim, Germany) and VWR (Darmstadt, Germany), respectively.

Samples and Methods

RPLC-RPLC Analysis of Polycyclic Aromatic Hydrocarbons (PAHs)

A mixture of 16 polycyclic aromatic hydrocarbons (PAHs) (PAH mix 25 from Dr. Ehrenstorfer, Augsburg, Germany (YA20950025AB)) was diluted with acetonitrile to obtain a concentration of 10 µg/mL of each compound. Table 1 shows the instrument parameters.

HILIC-RPLC Analysis of Peptides

A peptide standard (Agilent 10 Peptide Standard, lyophilized 71 µg, p/n 5190-0583) was dissolved in 100 µL water/acetonitrile (50/50, v/v) containing 0.1 % formic acid. Table 2 shows the instrument parameters.

The following peptides are contained in the peptide standard:

- Bradykinin frag 1-7 (Peptide 1)
- Bradykinin (Peptide 2)
- Angiotensin II (Peptide 3)
- Neurotensin (Peptide 4)
- Angiotensin I (Peptide 5)
- Renin substrate porcine (Peptide 6)
- [Ace-F-3,-2 H-1] Angiotensinogen (1-14) (Peptide 7)
- Ser/Thr Protein Phosphatase (15-31) (Peptide 8)
- [F14] Ser/Thr Protein Phosphatase (15-31) (Peptide 9)
- Melittin (Peptide 10)

Table 1. RPLC-RPLC parameters.

First dimension	
Column	Agilent ZORBAX Eclipse Plus PAH, 2.1 × 100 mm, 1.8 µm
Solvent	A) Water B) Methanol
Gradient	0 minutes – 50 %B 15 minutes – 100 %B Stop time: 22 minutes Post time: 5 minutes Stop time and post time were set in the ^{2}D pump to allow extension of the run time until completion of ^{2}D analysis of all cuts.
Flow rate	0.200 mL/min
Temperature	40 °C
Detection	DAD; 230 nm/4 nm, reference 390 nm/20 nm; 20 Hz To protect the flow cell from pressure pulses originating from valve switches, a pressure release kit (G4236-60010) was installed between the ^{1}D DAD and the 2D-LC valve.
Injection	Injection volume: 2 µL Sample temperature: 8 °C Needle wash: 3 seconds in water/acetonitrile (70/30)
Second dimension	
Column	Agilent ZORBAX Eclipse Plus C8, 2.1 × 50 mm, 1.8 µm
Solvent	A) Water B) Acetonitrile
Temperature	40 °C
Detection	DAD; 230 nm/4 nm, reference 390 nm/20 nm; 80 Hz
2D-LC	
2D-LC Mode	Heart-cutting
Flow rate	1.000 mL/min
Sampling table	Time based heart-cutting with a sampling time of 0.20 minutes was set up using the ^{1}D chromatogram of the PAH mixture as reference chromatogram.
ASM and ^{2}D gradient – Method without the use of ASM	
ASM	ASM disabled
^{2}D Gradient	0.00 minutes – 50 %B 1.00 minutes – 100 %B ^{2}D gradient stop time: 1.20 minutes ^{2}D cycle time: 1.70 minutes
ASM and ^{2}D gradient – Method with the use of ASM using an ASM factor of 1.5	
ASM	ASM capillary: 5500-1303 (0.12 × 680 mm) ASM enabled (ASM factor: 1.5) Flush sample loop 3.0 times (0.18 minutes)
^{2}D Gradient	0.00 minutes – 5 %B 0.18 minutes – 5 %B 0.19 minutes – 50 %B 1.19 minutes – 100 %B ^{2}D gradient stop time: 1.39 minutes ^{2}D cycle time: 1.89 minutes
ASM and ^{2}D gradient – Method with the use of ASM using an ASM factor of 2.0	
ASM	ASM capillary: 5500-1302 (0.12 × 340 mm) ASM enabled (ASM factor: 2.0) Flush sample loop 3.0 times (0.24 minutes)
^{2}D Gradient	0.00 minutes – 5 %B 0.24 minutes – 5 %B 0.25 minutes – 50 %B 1.25 minutes – 100 %B ^{2}D gradient stop time: 1.45 minutes ^{2}D cycle time: 1.95 minutes

Table 2. HILIC-RPLC parameters.

First dimension	
Column	Agilent ZORBAX HILIC Plus, 2.1 × 100 mm, 3.5 µm
Solvent	A) 50 mM ammonium formate in water adjusted to pH 4.0 B) Acetonitrile
Gradient	0 minutes – 85 %B 10 minutes – 60 %B 11 minutes – 60 %B 12 minutes – 85 %B Stop time: 12 minutes Post time: 15 minutes Stop time and post time were set in the ^{2}D pump to allow extension of the run time until completion of ^{2}D analysis of all cuts.
Flow rate	0.500 mL/min
Temperature	40 °C
Detection	DAD; 220 nm/4 nm, reference 360 nm/100 nm; 20 Hz To protect the flow cell from pressure pulses originating from valve switches, a pressure release kit (G4236-60010) was installed between the ^{1}D DAD and the 2D-LC valve.
Injection	Injection volume: 5 µL Sample temperature: 8 °C Needle wash: 3 seconds in water/acetonitrile (10/90)
Second dimension	
Column	AdvanceBio Peptide Mapping, 2.1 × 100 mm, 2.7 µm
Solvent	A) Water + 0.1 % formic acid B) Acetonitrile + 0.1 % formic acid
Temperature	40 °C
Detection	DAD and mass selective detection (MSD)
DAD	220 nm/4 nm, reference 360 nm/100 nm; 40 Hz
Mass selective detection (MSD)	
MSD Signals	Polarity: Positive Mode: Scan Mass range: 100–1,350 *m/z* Fragmentor: 100 V Gain: 1.00 Threshold: 150 Step size: 0.10 Peak width: 0.10 minutes Data storage: Full Stop time: No Limit The stop time was set to No Limit to allow extension of the run time until completion of ^{2}D analysis of all cuts.
Spray chamber	Drying gas flow: 10.0 L/min Nebulizer: 35 psig Drying gas temperature: 200 °C Sheath gas temperature: 300 °C Sheath gas flow: 10.0 L/min Capillary voltage: 2,500 V Nozzle voltage: 300 V

2D-LC	
2D-LC Mode	Heart-cutting
Flow rate	0.500 mL/min
Sampling table	1.00 minute time based with 0.08 minutes sampling time 4.00 minutes peak based with 0.15 minutes sampling time 6.50 minutes peak based with 0.25 minutes sampling time 8.00 minutes peak based with 0.15 minutes sampling time 8.50 minutes peak based with 0.45 minutes sampling time 10.00 minutes off
Peak detection	Mode: slope Upslope: 3 mAU/s Downslope: 1 mAU/s
ASM and ^{2}D gradient – Method without the use of ASM	
ASM	ASM disabled
^{2}D Gradient	0.00 minutes – 15 %B 3.00 minutes – 60 %B ^{2}D gradient stop time: 3.00 minutes ^{2}D cycle time: 5 minutes
ASM and ^{2}D gradient – Method with the use of ASM using an ASM factor of 3.0	
ASM	ASM capillary: 5500-1301 (0.12 × 170 mm) ASM enabled (ASM factor: 3.0) Flush sample loop 3.0 times (0.74 minutes)
^{2}D Gradient	0.00 minutes – 3 %B 0.74 minutes – 3 %B 0.75 minutes – 15 %B 3.75 minutes – 60 %B ^{2}D gradient stop time: 3.75 minutes ^{2}D cycle time: 5.75 minutes
ASM and ^{2}D gradient – Method with the use of ASM using an ASM factor of 5.1	
ASM	ASM capillary: 5500-1300 (0.12 × 85 mm) ASM enabled (ASM factor: 5.1) Flush sample loop 3.0 times (1.23 min)
^{2}D Gradient	0.00 minutes – 3 %B 1.23 minutes – 3 %B 1.24 minutes – 15 %B 4.24 minutes – 60 %B ^{2}D gradient stop time: 4.24 minutes ^{2}D cycle time: 6.24 minutes

Results and Discussion

The incompatibility between the ^{1}D and ^{2}D solvent systems is often referred to as one of the major challenges in 2D-LC method development[2]. ASM provides a valve-based approach to address this problem by diluting the ^{1}D effluent with ^{2}D mobile phase ahead of the ^{2}D column[2].

To enable ASM, a valve topology consisting of the 2D-LC Valve Active Solvent Modulation (G4243A) and two MHC valves (G4242-64000) is used. Figure 1 shows the valve configuration.

Figure 2 explains the principle of active solvent modulation by showing the valve switching cycle.

In position 1, a fraction from the ^{1}D is sampled in Deck A (MHC valve A). Meanwhile, the flow from the ^{2}D pump flows through Deck B (MHC valve B). Once the analysis of the fraction sampled in Deck A starts, the ASM valve switches to position 2. The flow coming from the ^{2}D pump enters the ASM valve at port 5, and is split inside the valve between Deck A (port 8) and the ASM capillary (port 6). At port 10, the ^{2}D flows through Deck A and the ASM capillary are reunited and led towards the ^{2}D column. In this valve position, the fraction of ^{1}D effluent that was sampled in Deck A is diluted with ^{2}D solvent before it reaches the ^{2}D column. Using weak ^{2}D solvent, focusing of the analytes at the head of the ^{2}D column is achieved. The ASM valve stays in position 2 until the fraction of ^{1}D effluent is entirely transferred to the ^{2}D (ASM phase). Once the transfer of the fraction is finished, the ASM valve switches to position 3. Now the entire flow coming from the ^{2}D pump flows through Deck A, and the ASM capillary is disconnected from the flow. The ASM valve stays in position 3 until the end of the ^{2}D analysis of the transferred fraction. Meanwhile, fractions from the ^{1}D effluent can be sampled on Deck B.

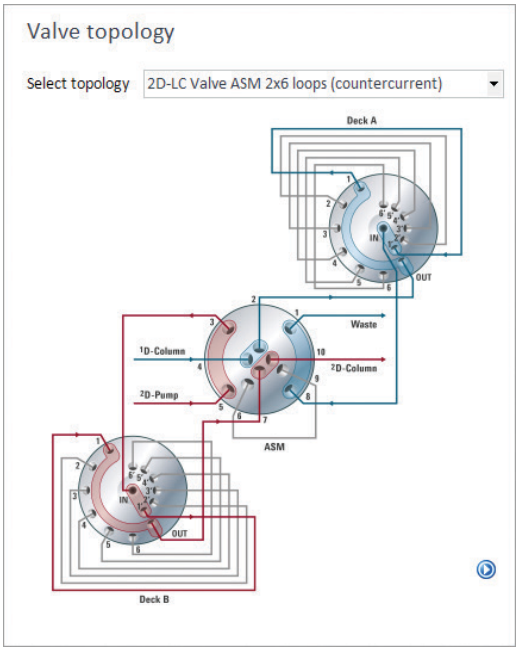


Figure 1. Valve topology for ASM, countercurrent configuration.

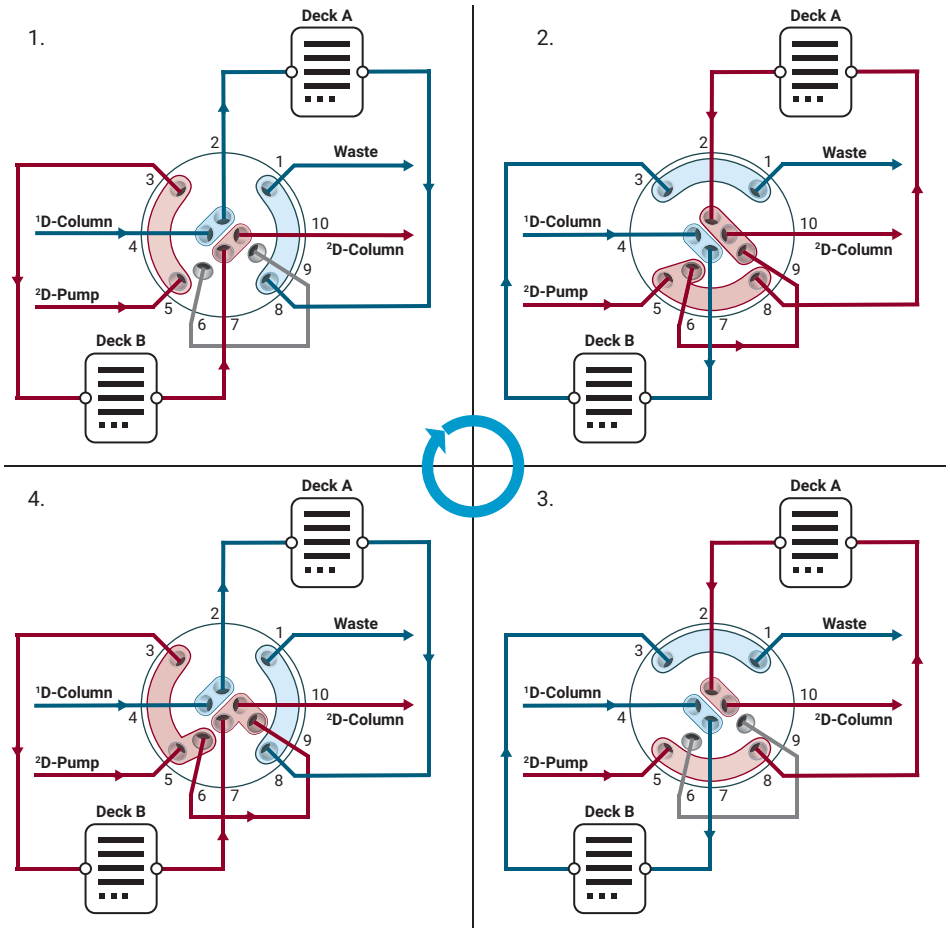


Figure 2. Valve switching cycle for ASM, countercurrent configuration.

Once the analysis of a fraction of ^{1}D effluent sampled in Deck B starts, the ASM valve switches to position 4 during the ASM phase to enable dilution of the ^{1}D effluent with ^{2}D solvent. After the ASM phase, the ASM valve switches back to position 1, and the ASM capillary is again disconnected from flow.

The ASM factor describes the dilution of the sampled fraction of ^{1}D effluent, which results from the split ratio between the deck and the ASM capillary. This split ratio depends on the backpressures generated at the ASM capillary and the flowpath through the deck. In the flowpath through the deck, the backpressure is mainly generated by the transfer capillaries between the ASM valve and the MHC valve. Different ASM capillaries are available to adjust the split ratio and the ASM factor (Table 3). If, for example, ASM capillary 5500-1302 (0.12 × 340 mm) is installed, it will generate the same backpressure as the transfer capillaries between the ASM valve and the MHC valve (two capillaries of 0.12 × 170 mm). Therefore, the flow from the ^{2}D pump is split with a ratio of 1:1 between the deck and the ASM capillary. This dilutes the sampled fraction of ^{1}D effluent by a factor of approximately 2 (ASM factor 2).

To use ASM, the ASM valve needs to be installed and configured in the 2D-LC configuration, as shown in Figure 1. The installed ASM capillary as well as transfer capillaries need to be defined in the 2D-LC configuration, as shown in Figure 3.

In the 2D-LC method, the use of ASM is activated and the length of the ASM phase is defined by specifying how many sample loop volumes are flushed through the loop to the ^{2}D in the valve position where dilution takes place (Figure 4). The resulting duration of the ASM phase is displayed. Flushing the sample loop three times is typically enough to achieve quantitative transfer and the recommended default.

Table 3. Available ASM capillaries.

ASM Capillary	Length (mm)	ID (mm)	Volume (µL)	ASM factor*	Split ratio* (loop: ASM)
5500-1300	85	0.12	0.96	5	1:4
5500-1301	170	0.12	1.9	3	1:2
5500-1302	340	0.12	3.8	2	1:1
5500-1303	680	0.12	7.7	1.5	1:0.5

* ASM factor and split ratio are based on transfer capillaries 5500-1270 (0.12 × 170 mm) and depend on solvent viscosity in the flowpaths through the ASM and transfer capillaries.

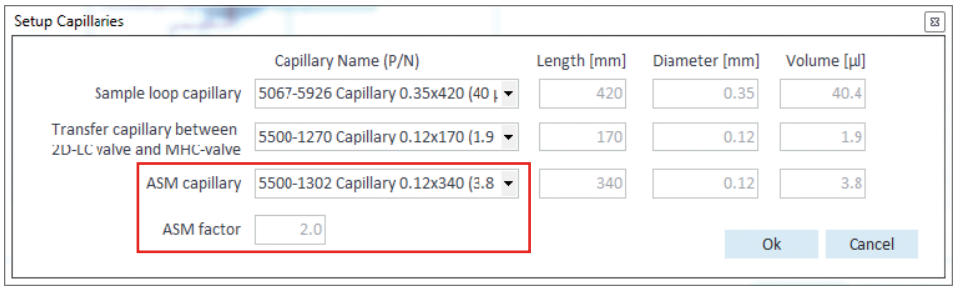


Figure 3. Configuration of the ASM capillary in the 2D-LC configuration.

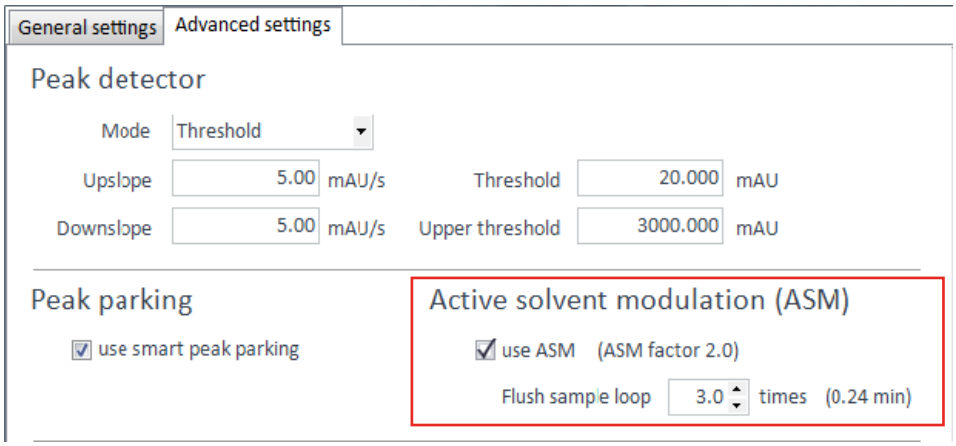


Figure 4. Activation of ASM and method setup in the 2D-LC method.

In the 2D-LC method, ^{2}D gradients that were initially programmed without the use of ASM need to be delayed by the duration of the ASM phase. The ^{2}D gradient stop time and ^{2}D cycle time need to be increased accordingly.

For effective use of ASM, a solvent composition with low strength is chosen during the ASM phase. Figure 5 demonstrates the change from a ^{2}D gradient initially programmed without the use of ASM (Figure 5A) to a ^{2}D gradient with the use of ASM using an ASM phase of 0.24 minutes duration (Figure 5B).

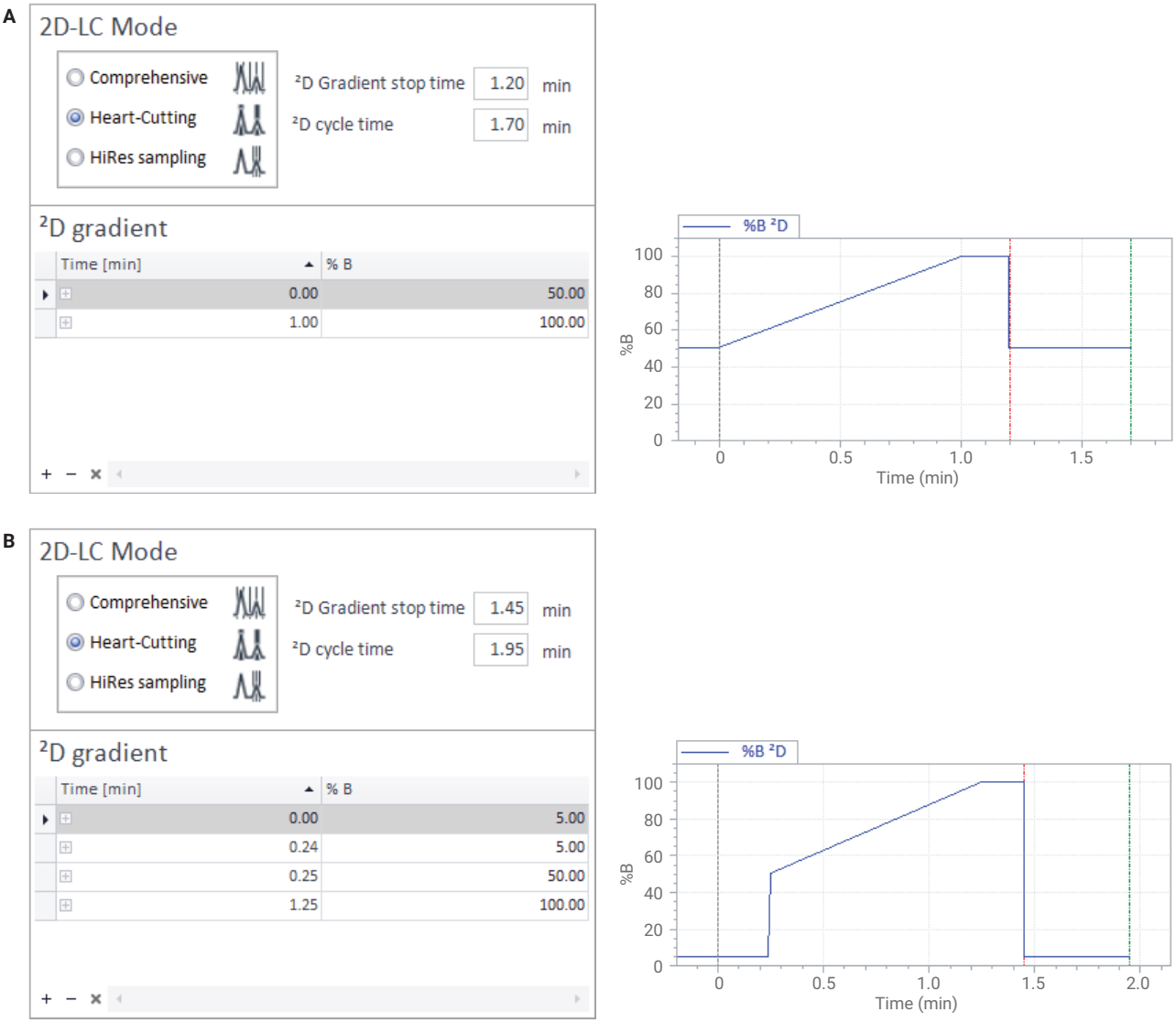


Figure 5. ^{2}D gradient setup. A) ^{2}D gradient initially programmed without the use of ASM. B) ^{2}D gradient with the use of ASM using an ASM phase of 0.24 minutes duration.

RPLC-RPLC Analysis of Polycyclic Aromatic Hydrocarbons (PAHs)

Figure 6 shows the MHC 2D-LC analysis of PAHs using RPLC in both dimensions. For this analysis, an Agilent ZORBAX Eclipse Plus PAH column was chosen as the ^{1}D column, and a ZORBAX Eclipse Plus C8 column was used in the ^{2}D. The retention of the PAHs is higher on the ZORBAX Eclipse Plus PAH column compared to the ZORBAX Eclipse Plus C8 column. Generally, it is beneficial for an RPLC-RPLC analysis to place the more retentive column in the ^{2}D to achieve focusing at the head of the ^{2}D column[2,3]. Here, the more retentive column was deliberately placed in the ^{1}D to show the challenge of this column order, and demonstrate that ASM enables focusing at the head of the ^{2}D column. ASM therefore improves ^{2}D resolution and sensitivity for this challenging combination.

As shown in Figure 6B, without the use of ASM, the ^{2}D PAH peaks are distorted due to the lack of focusing at the ^{2}D column head. This lack of focusing is because of the high percentage of organic solvent contained in the fractions of ^{1}D effluent transferred to the ^{2}D. Figures 6C and 6D demonstrate that ASM enables focusing at the ^{2}D column head, resulting in symmetrical and narrow ^{2}D peaks.

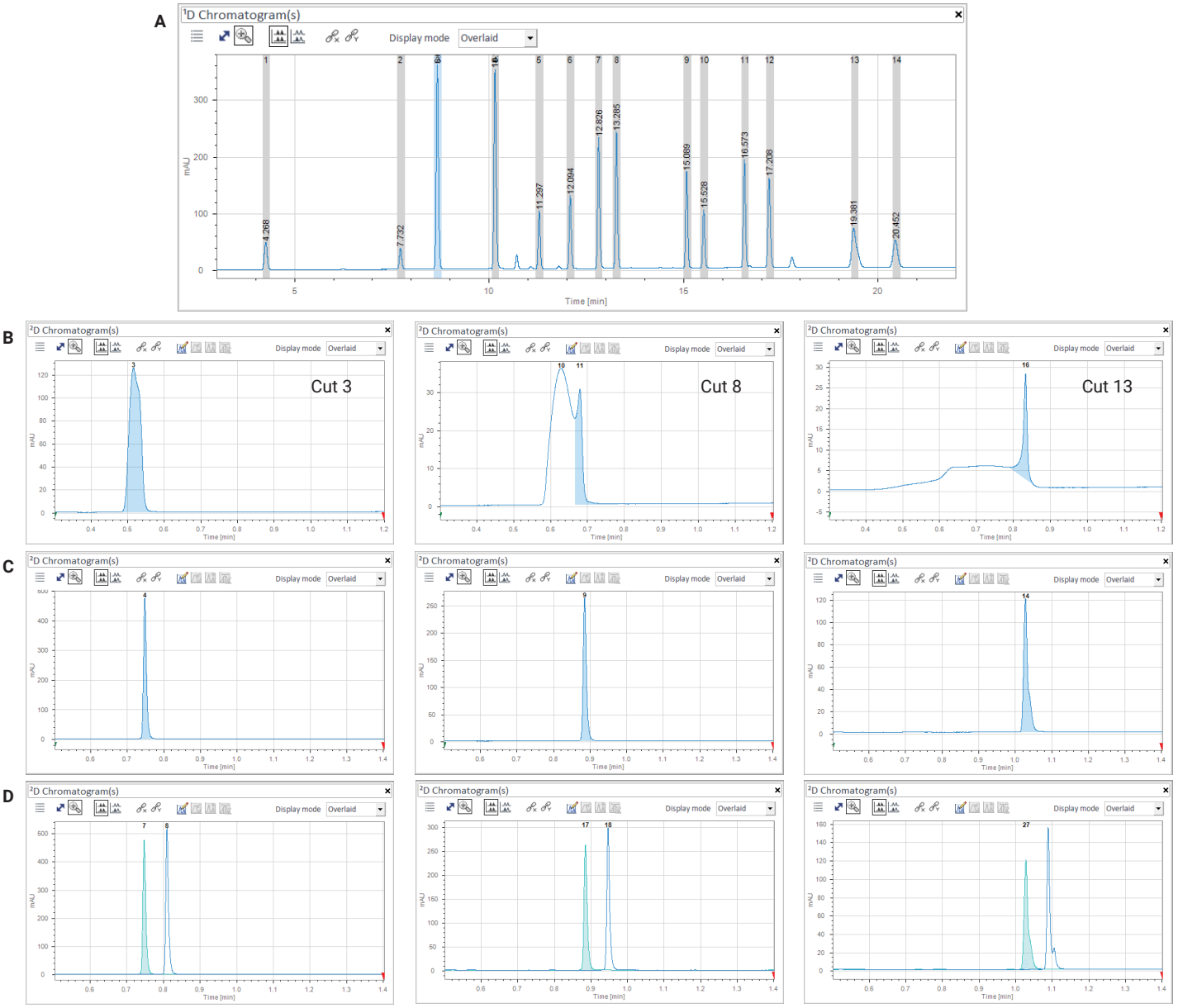


Figure 6. Multiple heart-cutting 2D-LC analyses of PAHs. A) ^{1}D chromatogram. B) ^{2}D Chromatograms from the analysis without the use of ASM. C) ^{2}D Chromatograms from the analysis with the use of ASM using an ASM factor of 1.5. D) ^{2}D Chromatograms from the analysis with the use of ASM using an ASM factor of 1.5 (green trace) and using an ASM factor of 2 (blue trace).

In Figure 6C, ASM was used with an ASM factor of 1.5, which leads to symmetrical and narrow ^{2}D peaks. Figure 6D compares the ^{2}D separations obtained using ASM with an ASM factor of 1.5 (green trace) and an ASM factor of 2 (blue trace). Due to the higher dilution of ^{1}D effluent enabled using an ASM factor of 2, a further reduction in ^{2}D peak width and increased peak height and sensitivity was obtained. In cut 13, a coelution that was already visible as a shoulder using an ASM factor of 1.5 was partially resolved using an ASM factor of 2.

To demonstrate that focusing at the ^{2}D column head depends on the dilution of ^{1}D effluent before the ^{2}D column and not solely on the ^{2}D gradient used, the analysis was performed using the same ^{2}D gradient with an initial hold at 5 %B (^{2}D gradient used during the analysis with an ASM factor of 2) with the use of ASM (Figure 7, green trace) and without the use of ASM (Figure 7, blue trace). The fact that symmetrical and narrow ^{2}D peaks are only obtained with the use of ASM clearly demonstrates the necessity of ASM to achieve focusing at the ^{2}D column head.

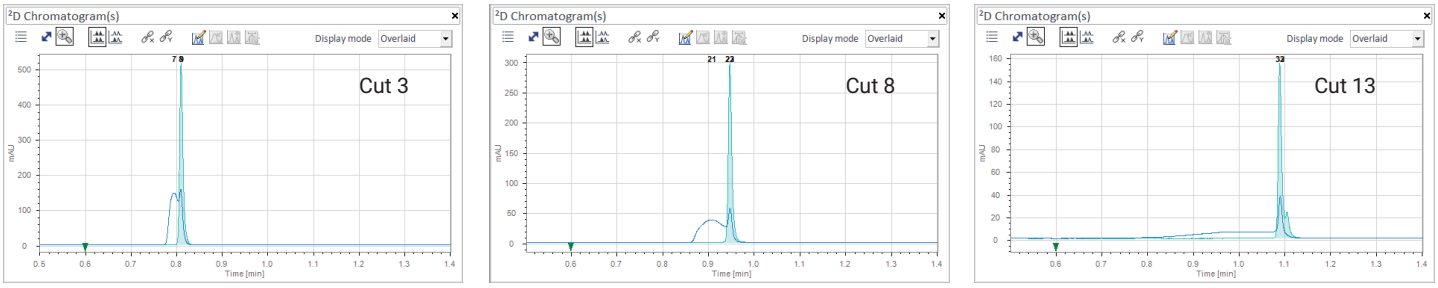


Figure 7. ^{2}D Chromatograms from the multiple heart-cutting 2D-LC analysis of PAHs using the same ^{2}D gradient with the use of ASM (green trace) and without the use of ASM (blue trace).

HILIC-RPLC Analysis of Peptides

Figure 8 shows the MHC 2D-LC analysis of peptides using HILIC in the ^{1}D and RPLC in the ^{2}D. In the ^{1}D chromatogram (Figure 8A), coelution of peptides is already visible for the later-eluting peaks. In Figures 8B–D, the ^{2}D extracted ion chromatograms (EICs) from the detected peptides are shown. Here, the most abundant ion detected for each peptide was extracted (for example, for peptide 9, EIC 1,050 was extracted, which corresponds to the $[M+2H]^{2+}$ ion of mol. wt. 2,099).

Figure 8B shows the analysis without the use of ASM. The peptides react differently to the high amount of organic solvent contained in the fractions of ^{1}D effluent transferred to the ^{2}D compared to the PAHs. The peptides show a partial breakthrough with the plug of ^{1}D effluent that was transferred to the ^{2}D column. The percentage of peptide retained on the ^{2}D column compared to the percentage that shows breakthrough is different for the different peptides analyzed. To confirm that the unretained peaks observed in the EICs originate from partial breakthrough of the peptides, the mass spectra of the

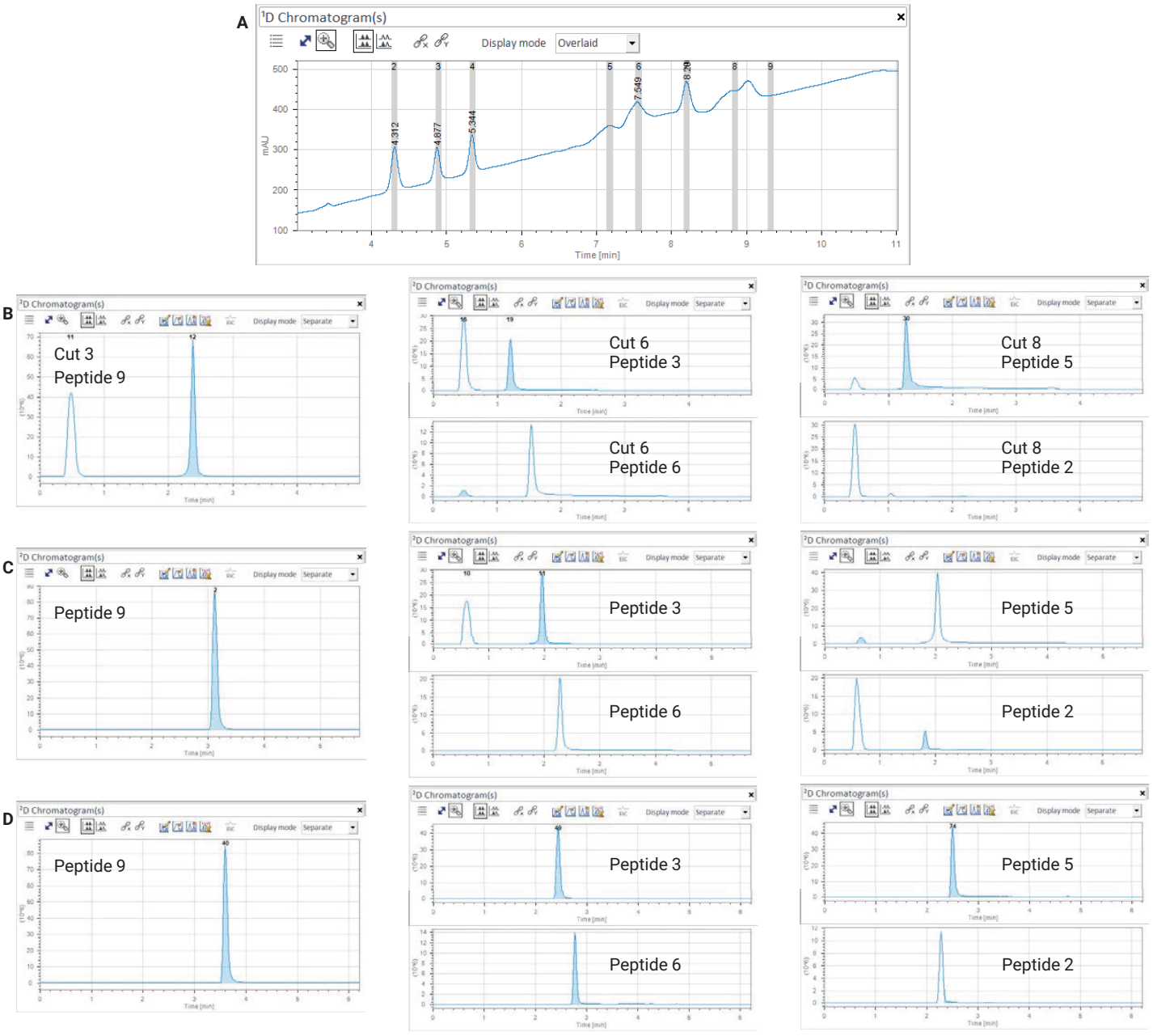


Figure 8. Multiple heart-cutting 2D-LC analyses of peptides: A) ^{1}D Chromatogram. B) ^{2}D EICs from the analysis without the use of ASM. C) ^{2}D EICs from the analysis with the use of ASM using an ASM factor of 3. D) ^{2}D EICs from the analysis with the use of ASM using an ASM factor of 5.

retained and unretained peaks were compared. The similarity of these mass spectra confirms the partial breakthrough. As an example, this is shown for cut 3 in Figure 9.

Figures 8C and 8D demonstrate that ASM enables complete retention of the peptides transferred to the ^{2}D, and avoids the partial breakthrough observed without the use of ASM. Because of the focusing at the ^{2}D column head, symmetrical ^{2}D peaks were obtained. In Figure 8C, ASM was used with an ASM factor of 3, which avoids breakthrough for certain peptides (for example, peptide 9 in cut 3). For other peptides (for example, peptide 3 in cut 6), an ASM factor of 3 did not provide sufficient dilution of the ^{1}D effluent with weak ^{2}D solvent to allow complete retention of the peptide. For these peptides, an ASM factor of 5 was necessary to completely avoid breakthrough, as shown in Figure 8D.

To demonstrate that avoiding breakthrough of the peptides depends on the dilution of ^{1}D effluent before the ^{2}D column and not solely on the ^{2}D gradient used, the analysis was performed using the same ^{2}D gradient with an initial hold at 3 %B (^{2}D gradient used during the analysis with an ASM factor of 5) with the use of ASM (Figure 10, green trace) and without the use of ASM (Figure 10, blue trace). Without the use of ASM, breakthrough of the peptides was observed, confirming the necessity of ASM to achieve retention of the peptides.

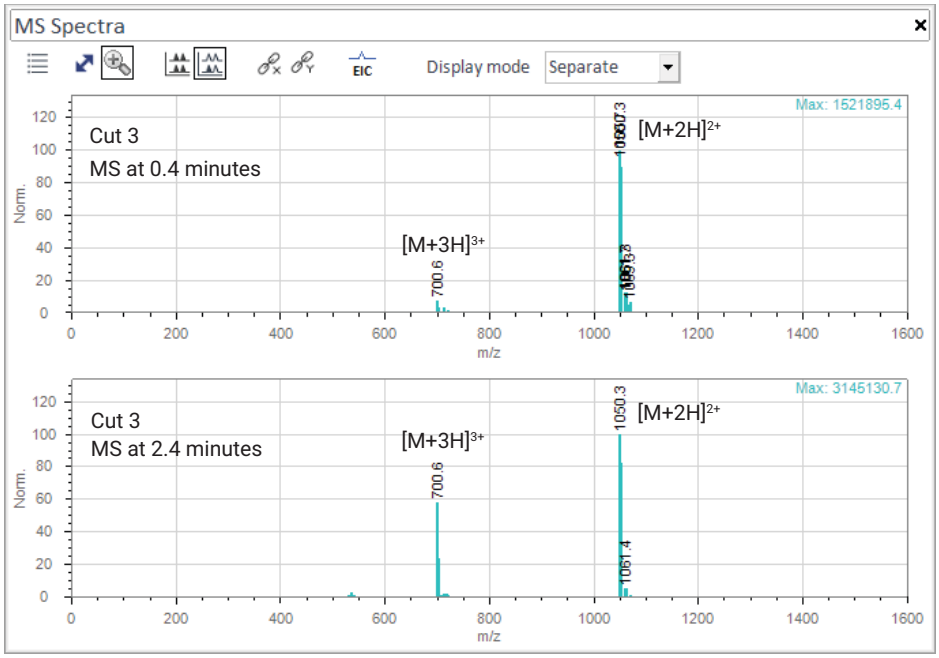


Figure 9. Mass spectra of the unretained (top) and retained (bottom) peak observed in cut 3 of the multiple heart-cutting 2D-LC analysis of peptides without the use of ASM.

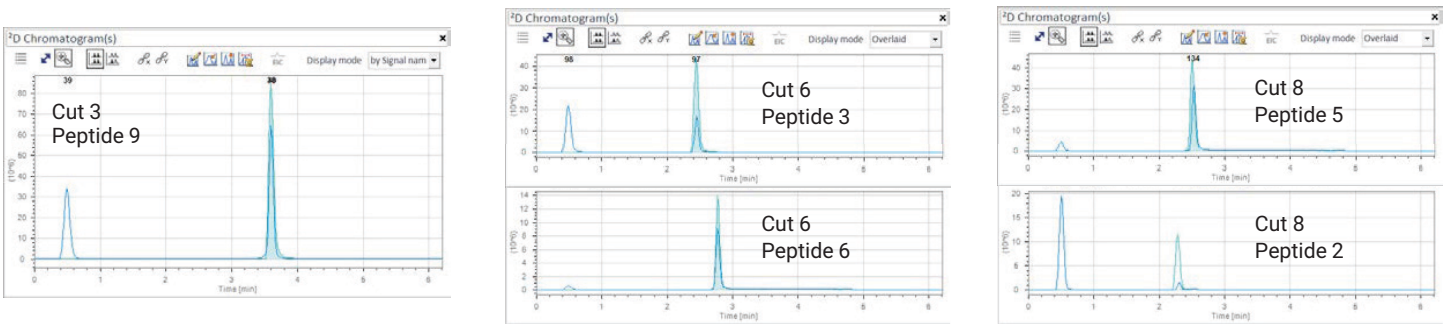


Figure 10. ^{2}D EICs from the multiple heart-cutting 2D-LC analysis of peptides using the same ^{2}D gradient with the use of ASM (green trace) and without the use of ASM (blue trace).

Conclusion

One major challenge in 2D-LC method development can be the incompatibility between the ^{1}D and ^{2}D solvent systems. For combinations of separation systems where the analytes show low retention on the ^{2}D column in the ^{1}D effluent, volume overload effects such as breakthrough or broad and distorted peaks in the ^{2}D can be observed. Active solvent modulation provides a valve-based approach to address this problem by diluting the ^{1}D effluent with ^{2}D mobile phase ahead of the ^{2}D column, thereby enabling focusing at the ^{2}D column head.

For an RPLC-RPLC analysis of PAHs, broad and distorted ^{2}D peaks were observed without the use of ASM. Using ASM with an ASM factor of 2 enabled focusing at the ^{2}D column head, leading to symmetrical and narrow ^{2}D peaks. During an HILIC-RPLC analysis of peptides, partial breakthrough of the peptides was observed in the ^{2}D without the use of ASM. Complete retention of the peptides was achieved using ASM with an ASM factor of 5.

References

1. Stoll, D. R.; Carr, P. W. Two-Dimensional Liquid Chromatography: A State of the Art Tutorial. *Analytical Chemistry* **2017**, *89*, 519-531.
2. Stoll, D. R.; *et al.* Active Solvent Modulation: A Valve-Based Approach To Improve Separation Compatibility in Two-Dimensional Liquid Chromatography. *Analytical Chemistry* **2017**, *89*, 9260-9267.
3. Li, D.; Jakob, C.; Schmitz, O. Practical considerations in comprehensive two-dimensional liquid chromatography systems (LCxLC) with reversed-phases in both dimensions. Analytical and *Bioanalytical Chemistry* **2015**, *407*, 153-167.

www.agilent.com/chem

Printed in the USA, December 1, 2017
5991-8785EN

Agilent
Trusted Answers