Application Note

Pharma and Biopharma

Agilent
Trusted Answers

Analysis of Peptone Samples Using the Agilent InfinityLab 2D-LC Solutions with Active Solvent Modulation

Authors

Gerd Vanhoenacker,
Pat Sandra, and Koen Sandra
Research Institute for
Chromatography (RIC)
President Kennedypark 26
B-8500 Kortrijk
Belgium

Sonja Krieger, Sonja Schneider,
and Udo Huber
Agilent Technologies, Inc.

Abstract

The Application Note demonstrates the use of an Agilent 1290 Infinity II 2D-LC system with Active Solvent Modulation (ASM) for a selection of peptone samples. Multiple heart-cutting 2D-LC and high-resolution sampling 2D-LC were carried out using a combination of size exclusion chromatography (SEC) and reversed-phase LC (RPLC) in the first and second dimension, respectively. Detection was performed with DAD and with MS. The ASM option proved to be valuable to maintain chromatographic performance and avoid breakthrough in the second dimension. Injection precision was evaluated for both 2D-LC modes, and peak area RSD values of less than 5 % were obtained. A selection of observed differences between various batches of peptone research samples is presented.

Introduction

Peptones are water-soluble sources of predominantly amino acids and peptides supplemented to chemically defined growth media to support cell growth and recombinant protein production. They result from the enzymatic or chemical hydrolysis (acid) of natural products such as milk (casein), soybean, wheat, and so forth. Batch-to-batch variability associated with peptones often leads to productivity differences. Therefore, there is a clear need for analytical methods to study the molecular basis for these differences, and to determine the quality of the peptones before they are used in biopharmaceutical manufacturing.

Peptones are characterized by an inherent structural complexity, and an ideal tool to handle such complex samples is two-dimensional liquid chromatography (2D-LC). In 2D-LC, the sample is subjected to two different separation mechanisms with orthogonal behavior, with the aim to increase the peak capacity. This Application Note describes the analysis of different casein hydrolysates using the Agilent 1290 Infinity II 2D-LC system. SEC and RPLC are used in the first (^{1}D) and second dimension (^{2}D), respectively. Using multiple heart-cutting or high-resolution sampling, ^{1}D cuts or snips are stored in 40 µL loops and individually transferred to the second dimension. To cope with the solvent strength incompatibility between the two dimensions (the SEC mobile phase contains 30 % acetonitrile), the ^{1}D effluent is diluted with a weak solvent before transfer to the ^{2}D column, a principle known as ASM.

Experimental

Instrumentation

An Agilent 1290 Infinity II 2D-LC system equipped with the multiple heart-cutting option was used. The configuration is summarized in Table 1. Detection of the first-dimension SEC was done with DAD. The second-dimension RPLC was monitored by DAD or MS. Further 1D-LC RPLC experiments were carried out using part of the configuration described in Table 1.

Mass selective detection (MSD) was performed using an Agilent 6130 single quadrupole LC/MS (G6130BA) equipped with an ESI source (G1948B). The column effluent was split using a zero dead-volume T-piece. A 0.075 × 220 mm capillary (5067-4787) was used to connect one side of the T-piece to the DAD, and a 0.075 × 340 mm capillary (5067-4783) to connect the other side directly to the MS source inlet (MS diverter valve was bypassed).

Software

- Agilent OpenLab CDS ChemStation revision C.01.07 SR4 [505]
- 2D-LC software revision A.01.04 [025]

Methods

Tables 2 and 3 summarize the method parameters.

Samples and sample preparation

Two types of samples were analyzed: one commercial casein hydrolysate sample (part number 22090) purchased from Merck (St. Louis, MO, USA) and three research casein hydrolysate peptone samples. Samples were dissolved in the SEC mobile phase (0.1 % TFA in 30 % acetonitrile) at 5 mg/mL. The SEC mobile phase was also used as a blank.

Table 1. 1D-LC and 2D-LC system configuration.

1D-LC	
Column	Agilent ZORBAX Eclipse Plus C18 RRHD, 3.0 × 100 mm, 1.8 µm (p/n 959758-302)
G7120A	Agilent 1290 Infinity II High Speed Pump
G7167B	Agilent 1290 Infinity II Multisampler
G7116B	Agilent 1290 Infinity II Multicolumn Thermostat
G7117B	Agilent 1290 Infinity II Diode Array Detector with a 10 mm Max-Light Cartridge Cell (p/n G4212-60008)
2D-LC	
^{1}D: SEC	
Column	Agilent AdvanceBio SEC 130 Å, 4.6 × 300 mm, 2.7 µm (p/n PL1580-5350)
G7120A	Agilent 1290 Infinity II High Speed Pump
G7167B	Agilent 1290 Infinity II Multisampler
G7116B	Agilent 1290 Infinity II Multicolumn Thermostat
G7117B	Agilent 1290 Infinity II Diode Array Detector with a 3.7 mm HDR Max-Light Cartridge Cell (p/n G4212-60032)
^{2}D: RPLC	
Column	Agilent ZORBAX Eclipse Plus C18 RRHD, 3.0 × 50 mm, 1.8 µm (p/n 959757-302)
G7120A	Agilent 1290 Infinity II High Speed Pump
G7116B	Agilent 1290 Infinity II Multicolumn Thermostat
G7117B	Agilent 1290 Infinity II Diode Array Detector with a 10 mm Max-Light Cartridge Cell (p/n G4212-60008)

Multiple Heart Cutting Option	
G1170A	Agilent 1290 Infinity Valve Drive with 2D-LC Valve, Active Solvent Modulation (p/n G4243A)
G1170, 2x	Two Agilent 1290 Infinity Valve Drives with Multiple heart-cutting Valves (p/n G4242-64000) equipped with 40 µL loops

Table 2. 1D-LC method parameters.

Column	Agilent ZORBAX Eclipse Plus C18 RRHD, 3.0 × 100 mm, 1.8 µm
Mobile Phase	A) 0.1 % phosphoric acid in water B) Acetonitrile
Flow Rate	0.5 mL/min
Gradient	Time (min) %B 0–2 2 2–37 2–47 37–38 47–95 38–45 95 6 minutes post-time at 2 %B
Temperature	35 °C
Injection	2 µL, needle wash 3 seconds with water/acetonitrile 50/50
Detection DAD	214/4, 280/4, 225/4 nm, reference 400/80 nm, peak width >0.025 minutes (10 Hz)

Table 3. 2D-LC method parameters.

	First Dimension (^{1}D)
Column	Agilent AdvanceBio SEC 130 Å, 4.6 × 300 mm, 2.7 µm
Mobile Phase	Water/acetonitrile 70/30 with 0.1 % TFA, isocratic
Flow Rate	0.2 mL/min
Temperature	23 °C
Injection	20 µL, needle wash 3 seconds with water/acetonitrile 50/50
Detection DAD	214/4, 280/4, 225/4 nm, reference 400/80 nm, peak width >0.05 minutes (5 Hz)
	Second Dimension (^{2}D)
Column	Agilent ZORBAX Eclipse Plus C18 RRHD, 3.0 × 50 mm, 1.8 µm
Mobile Phase	A) 0.1 % phosphoric acid in water (for DAD), 0.1 % formic acid in water (for MS) B) Acetonitrile
Temperature	35 °C
Detection DAD	214/4, 280/4, 225/4 nm, reference 400/80 nm, peak width >0.013 minutes (20 Hz)
Detection MS	**MS source** Ionization mode ESI Drying gas temperature 340 °C Drying gas flow 11 L/min Nebulizer pressure 45 psig Capillary voltage 3,500 V **MS acquisition** Polarity Positive Scan 100–1,750 *m/z*, step size 0.15 Fragmentor Ramped: 75 V at 100 *m/z*, 210 V at 1,750 *m/z* Peak width 0.05 minutes

	2D-LC
Flow Rate	1.2 mL/min (idle flow rate 0.3 mL/min)
ASM	ASM capillary p/n 5500-1300 (0.12 × 85 mm) ASM factor 5.1 Flush sample loop 3.0 times (0.51 minutes)
Gradient	Time (min) %B 0–0.51 2 ASM phase 0.51–5.51 2–47 5.51–6.10 47–95 6.10–7.50 95 ^{2}D gradient stop time 7.50–10.00 2 ^{2}D cycle time
Sampling	**Multiple heart-cutting** • Time based with 0.2 minutes sampling time • 13.05, 13.80, 14.93, 16.61 minutes **High-resolution sampling** • Time based with 9.5 seconds sampling time • 15.60 minutes, 4 cuts

Results and discussion

1D-LC

The first experiments served to characterize the different samples and assess their complexity. Therefore, 1D-LC experiments were carried out with both LC modes. 1D-LC SEC results were obtained from the first dimension of the 2D-LC analyses with the DAD installed before the 2D-LC valve. The RPLC results were generated with an alternative column and method. This separation was not fully optimized, but the goal was to have a higher peak capacity compared to the second-dimension separation to visualize the complexity of the samples at hand. To realize this, a longer column with the same packing and internal diameter was used at a lower flow rate, with a shallower gradient.

Figure 1 shows the results of these 1D-LC analyses for the casein hydrolysate samples. From the SEC results, it can be concluded that the commercial sample contains a significantly larger low molecular weight fraction (higher retention time). The hydrolysis in this peptone product apparently was more complete compared to the research samples. This is also reflected in the RPLC result, where the commercial sample shows a less complex profile compared to the research samples. Some abundant peaks are observed in the first part of the chromatogram, whereas for the research sample, the peaks are more scattered across the entire gradient. Upon comparing the different peptone research samples (bottom chromatograms of Figure 1) some differences can be observed.

2D-LC and ASM

One way to overcome the lack of resolution and peak capacity for these complex samples is to carry out multidimensional LC. 2D-LC is known to greatly enhance peak capacity and resolving power.[1] Some of the prerequisites, however, are that both dimensions are compatible with and orthogonal to each other, and that the separation efficiency in both dimensions is not reduced significantly by the 2D-LC process.

For this particular analysis, a multiple heart-cutting 2D-LC approach was developed where SEC was installed in the first dimension and RPLC in the second dimension. To reduce the overall analysis time, the column in the second dimension was shorter compared to the column used in the 1D-LC setup.

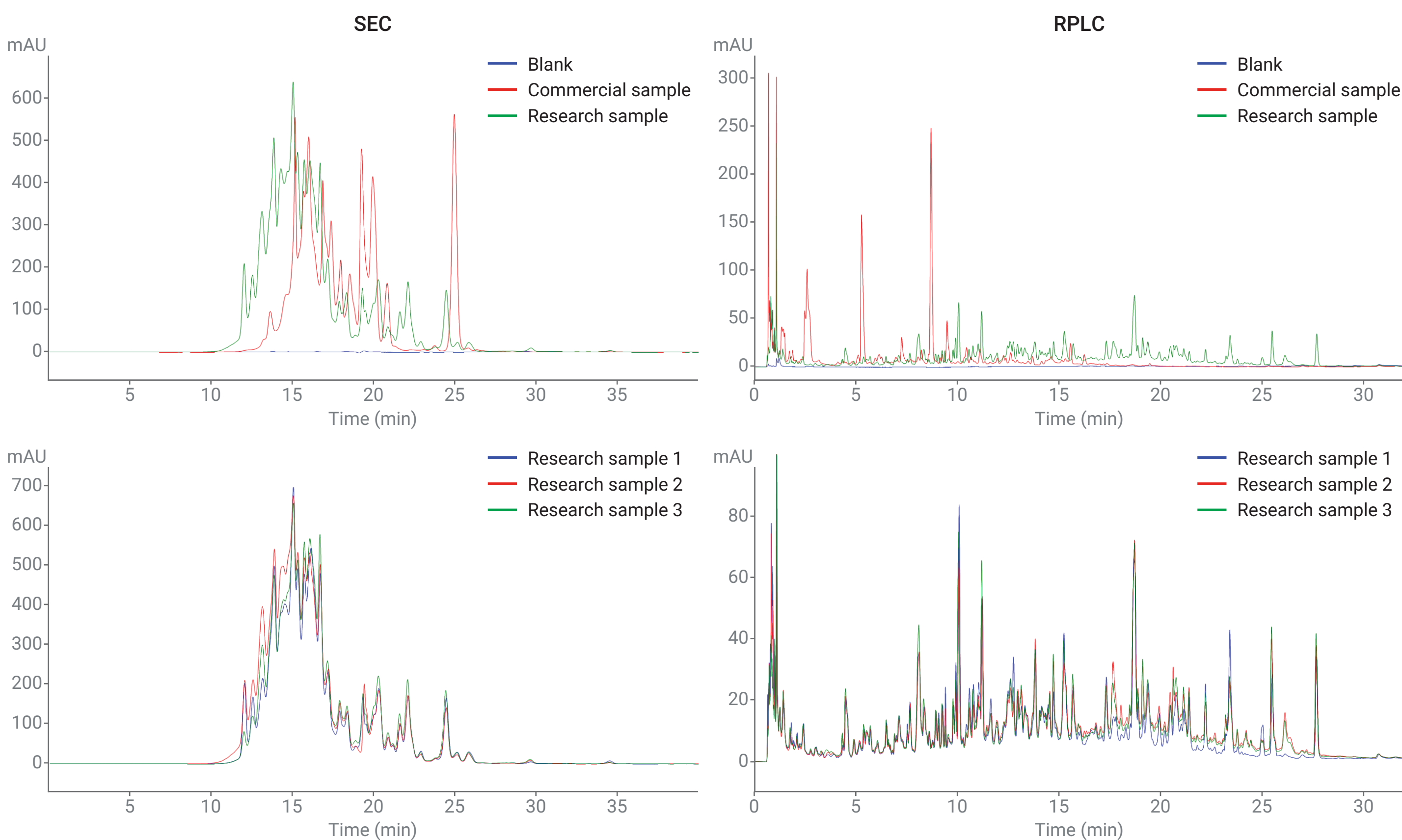


Figure 1. 1D-LC analyses of a blank solution and a commercial and a research peptone sample (top), and 1D-LC comparison of the three research peptone samples (bottom). Detection: DAD, 214 nm.

In this Application Note, orthogonality between SEC (separation predominantly based on molecular size or volume) and RPLC (separation based on hydrophobicity) is definitely present. This was also indicated by the 1D-LC SEC and RPLC results. The mobile phases show good compatibility (both are water/acetonitrile mixtures with additives).

The performance of the (^{2}D) RPLC separation is, however, severely impacted in the 2D-LC process. The reason can be found in the fact that in the setup a large volume (40 µL) of the SEC mobile phase (0.1 % TFA in 30 % acetonitrile) is injected on the RPLC column. The combination of the acetonitrile in the injection solvent and the large volume introduced results in poor chromatographic performance for the more polar solutes in the peptone samples in RPLC.

The ASM valve was designed to relieve the adverse effects of large volume transfer from the ^{1}D to the ^{2}D. ASM dilutes the content of the sampling loop containing sample and ^{1}D solvent with weak ^{2}D solvent before it reaches the second-dimension column, and therefore improves the separation in the second dimension. This dilution is achieved by a parallel flow of solvents through the sample loop and an ASM capillary. The length and internal diameter of the ASM capillary determine the dilution factor. A graphical representation of the ASM valve (displayed in the position during sample transfer from the ^{1}D to the ^{2}D) is presented in Figure 2. More information about the principle is described in the work of Stoll; *et al.*[2] and in Technical Overview 5991-8785EN.[3]

Figure 3 illustrates the impact of the ASM principle for a research peptone sample. Upon running the multiple heart-cutting method without ASM (the ASM function is disabled in the software) a large, sharp peak is visible at the start of each second-dimension analysis. This originates from sample breakthrough due to the lack of retention of polar substances on the second-dimension column. The large fraction transferred contains 30 % acetonitrile, which makes it very difficult to retain these compounds, so they are eluted with the second-dimension void volume.

When ASM is activated, a clear increase in signal intensity is observed for the early eluting compounds. This indicates that the breakthrough is significantly reduced, and full recovery for these compounds is achieved. Later eluting compounds are less affected by the breakthrough since their retention on the column is large enough to overcome the issues related to injection solvent strength and volume. Using ASM, a relatively large and broad peak is observed shortly after the second-dimension void time. This peak is very likely caused by the TFA present in the first-dimension mobile phase. The fact that it is also present in blank analyses (Figure 3) proves that the signal is not related to breakthrough of peptone compounds.

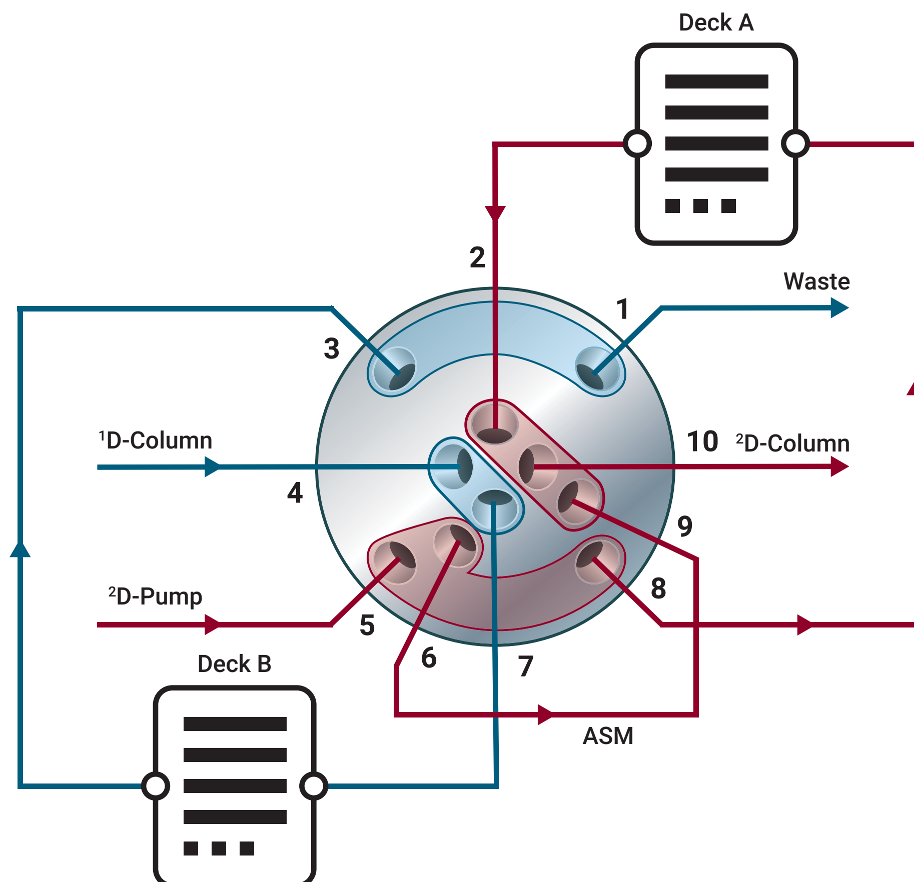


Figure 2. Valve topology for ASM.

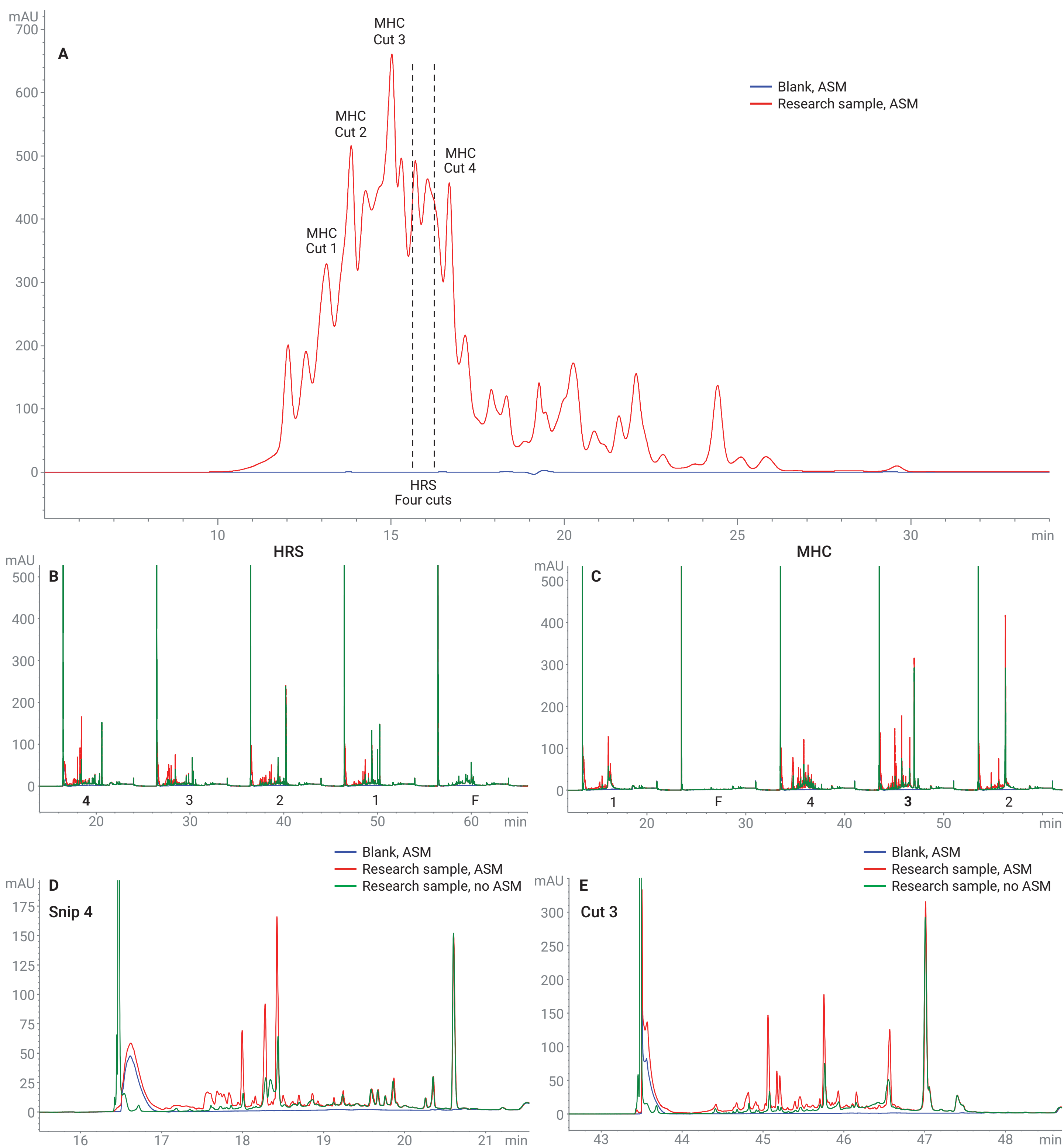


Figure 3. 2D-LC analyses of a blank solution and a research peptone sample using ASM and a research peptone sample with ASM disabled. (A) [1]D result with the sampling regions indicated. (B)–(E) [2]D results: left panels show results for high-resolution sampling: full [2]D (B), and detail for snip 4 (D). Right panels show results for multiple heart-cutting, full [2]D (C), and detail for cut 3 (E). Detection: DAD, 214 nm.

Breakthrough and loss of signal is not the only problem generated by large volume injection. Severe peak distortion was also observed. To demonstrate this, Figure 4 shows the impact of ASM in a 2D-LC/MS experiment. The extracted ion chromatogram (EIC) for a selection of peaks in a research peptone sample is plotted for an analysis with and without ASM. As observed before, signal intensity is greatly enhanced with ASM, indicating less or no breakthrough. The breakthrough peak, when ASM is disabled, is less obvious in the MS data, probably due to ionization suppression caused by the TFA present in the ^{1}D mobile phase (MP). The adverse effect of TFA on ionization in MS is a well known phenomenon.[4,5] The peak shapes for many of the compounds shown here are not severely affected by the breakthrough. The signal for ion 326.8 *m/z*, however, shows significant peak fronting when ASM is not used. Upon activating the ASM option, this problem is resolved.

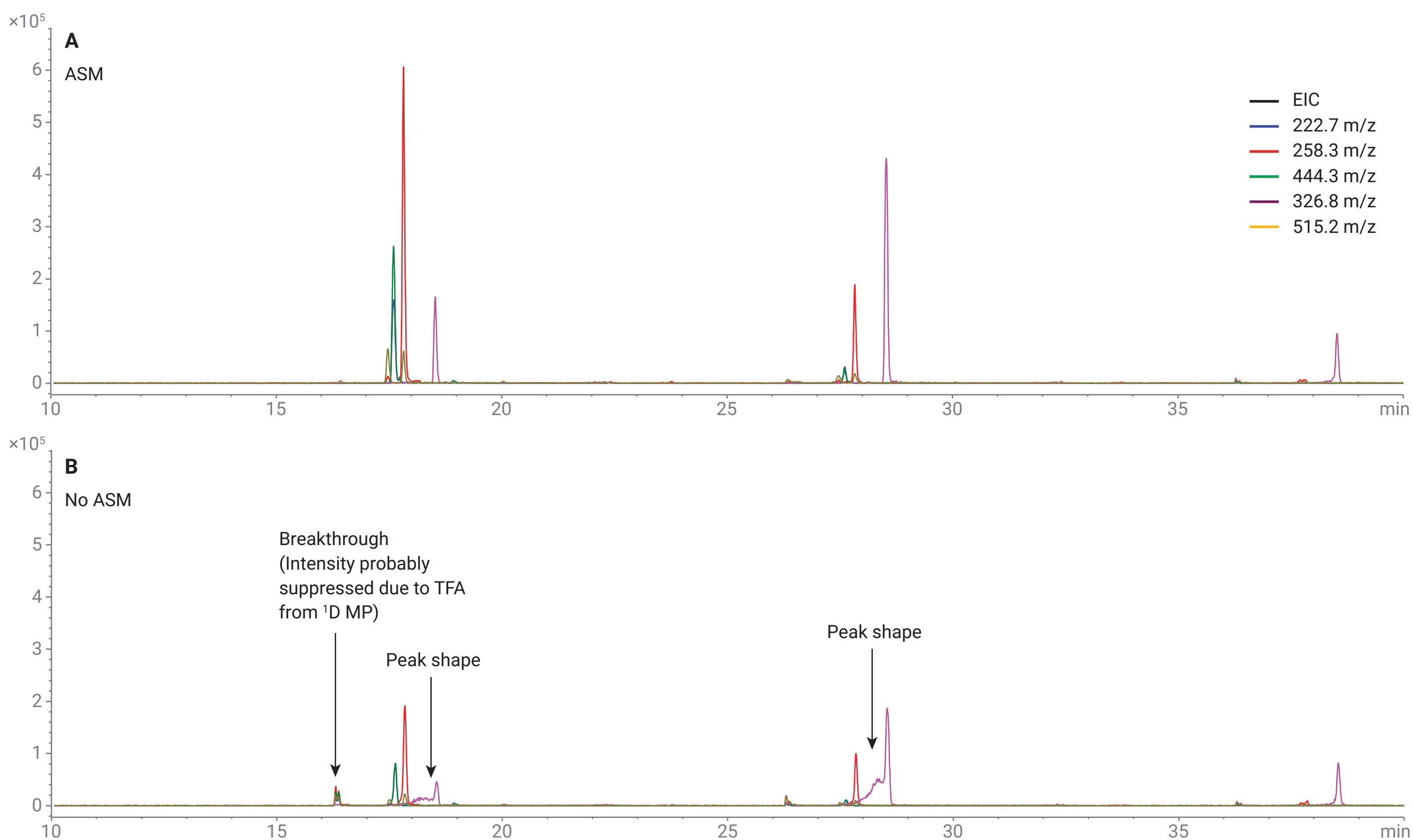


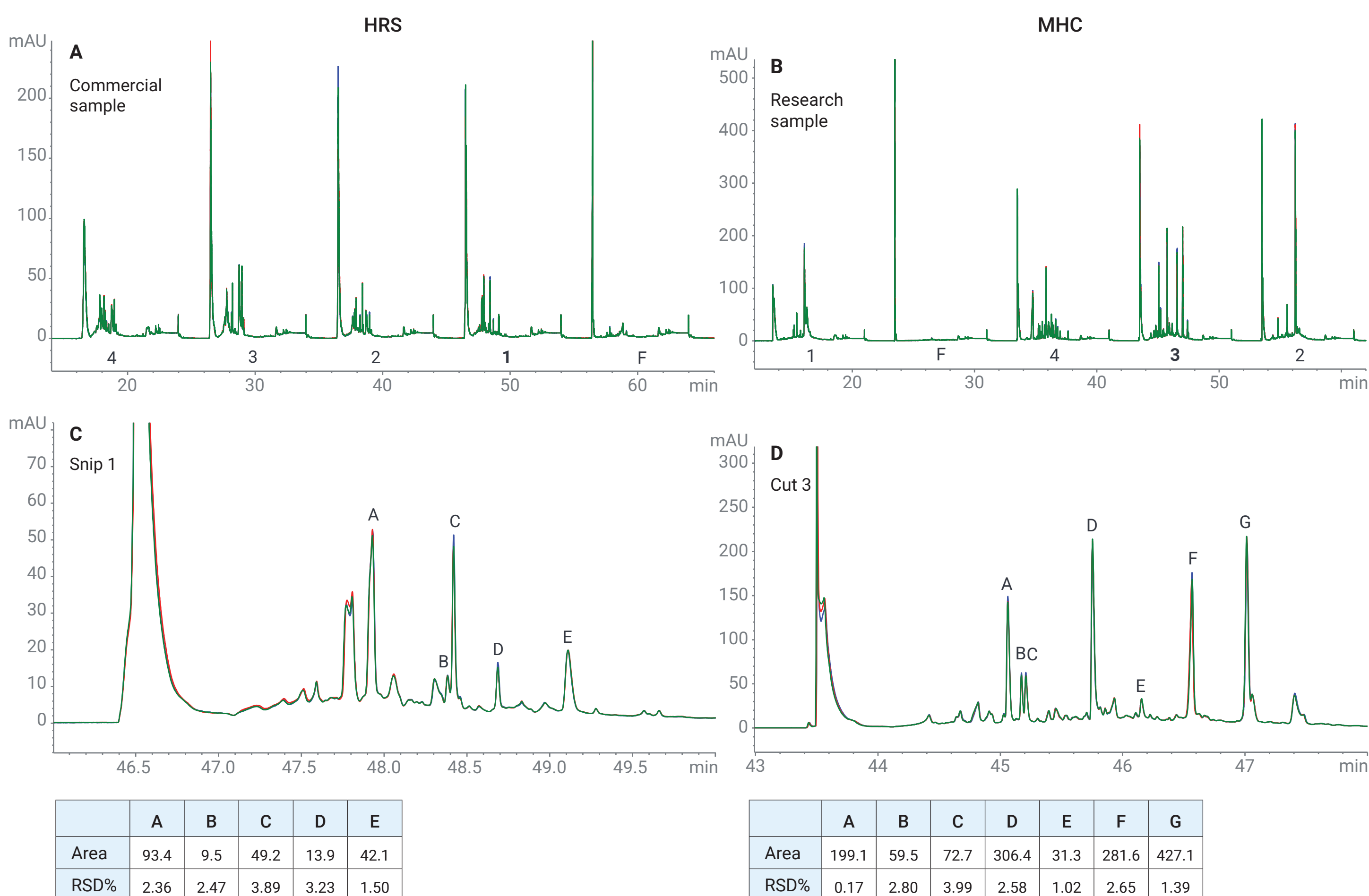
Figure 4. Impact of ASM on the peak intensity and peak shape. EIC for a selection of ions for 2D-LC analysis (high-resolution sampling) of a research peptone sample is shown.

The injection precision and repeatability of the 2D-LC procedure of both methods was evaluated by triplicate analysis using DAD. Excellent retention time repeatability is achieved in both dimensions. This is also translated into the good precision obtained in the second-dimension peak area for a selection of peaks. Figure 5 shows the RSD% values for both multiple heart-cutting and high-resolution sampling together with a detail of the ²D chromatograms.

Although some differences between the research samples could be observed in the 1D-LC analyses, it was impossible to obtain a quantitative estimate of the differences because of the complexity of the chromatograms. Applying a 2D-LC approach greatly simplifies the chromatograms since only a small fraction of the sample is transferred to the second-dimension RPLC separation. This enables a detailed look into a particular SEC region.

The following example demonstrates observations that can be made in the 2D-LC-DAD analyses. The relatively crowded ²D chromatograms indicate that even a very small fraction of the SEC effluent generates a large number of peaks in RPLC, illustrating the complexity of the samples at hand. Even though the number of peaks in the second-dimension chromatograms is significant, it is possible to isolate differences between the research samples. In the examples shown in the figures, some differences detected with a DAD were further investigated with MS.

Figure 6 shows the use of the high-resolution sampling approach for comparison of the research peptone samples. The 2D-LC approach visualizes differences between samples, and enables integration and quantitation of these differences with DAD. Supporting MS data indicate that, in this case, the compound has an *m/z* of 449.8.



	A	B	C	D	E
Area	93.4	9.5	49.2	13.9	42.1
RSD%	2.36	2.47	3.89	3.23	1.50

	A	B	C	D	E	F	G
Area	199.1	59.5	72.7	306.4	31.3	281.6	427.1
RSD%	0.17	2.80	3.99	2.58	1.02	2.65	1.39

Figure 5. Overlay of 2D-LC analyses of three consecutive injections of peptone samples using ASM. Left panels show results for high-resolution sampling, full ²D (A) and detail for snip 1 (C). Right panels show results for multiple heart-cutting, full ²D (B) and detail for cut 3 (D). Detection: DAD, 214 nm.

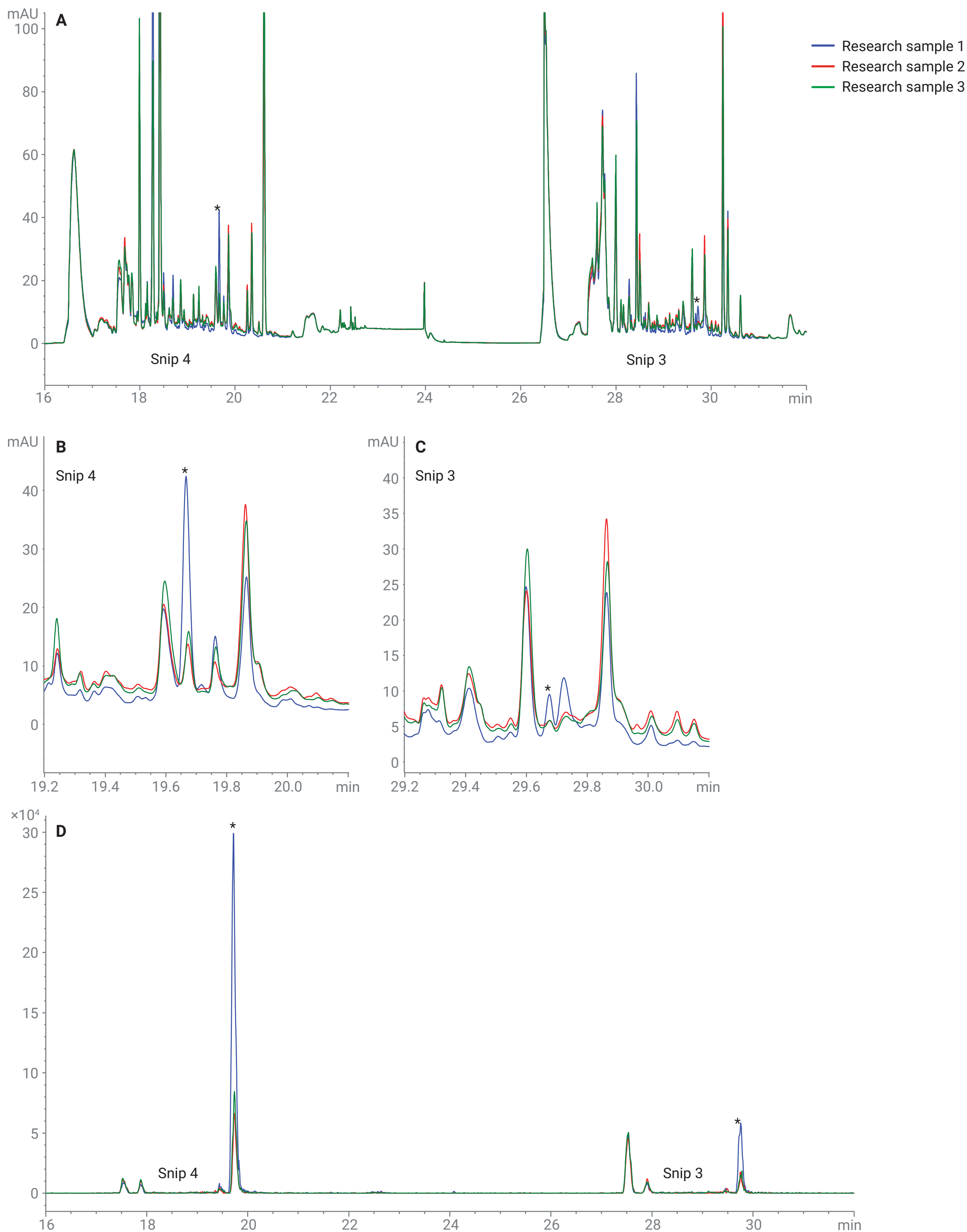


Figure 6. Comparison of high-resolution sampling analyses of the three research samples. (A) Second-dimension DAD signal for snips 3 and 4; (B),(C) detail of DAD signals; (D) second-dimension MS signal (EIC *m/z* 449.8) for snips 3 and 4.

Conclusion

This 2D-LC method allows a detailed analysis of complex peptone samples. Both the multiple heart-cutting 2D-LC and high-resolution sampling 2D-LC were evaluated for injection precision and sample analysis. Repetitive injections demonstrate the retention time and peak area stability in both dimensions.

The key for maintaining excellent chromatographic performance lies in the 2D-LC system with ASM that adequately focuses the transferred fraction onto the second-dimension column. This allows the high peak capacity required for this detailed analysis to be maintained.

References

1. Vanhoenacker, G.; *et al.* Increasing Peak Capacity Using the Agilent 1290 Infinity 2D-LC Solution. *Agilent Technologies Technical Overview*, publication number 5991-6123EN, **2015**.
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. Krieger, S. The Agilent InfinityLab 2D-LC Solution with Active Solvent Modulation. *Agilent Technologies Technical Overview*, publication number 5991-8785EN, **2017**.
4. Apffel, A.; *et al.* Enhanced Sensitivity for Peptide Mapping with Electrospray Liquid Chromatography-Mass Spectrometry in the Presence of Signal Suppression due to Trifluoroacetic Acid-Containing Mobile Phases. *Journal of Chromatography A* **1995**, *712*, 177–190.
5. Kostiainen, R.; Kauppila, T. J. Review: Effect of Eluent on the Ionization Process in Liquid Chromatography-Mass Spectrometry. *Journal of Chromatography A* **2009**, *1216*, 685–699.

www.agilent.com/chem

This information is subject to change without notice.

© Agilent Technologies, Inc. 2019

Printed in the USA, May 1, 2019
5994-0926EN

Agilent
Trusted Answers