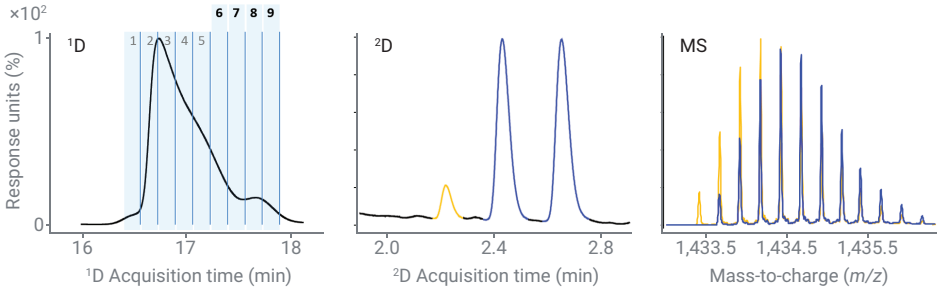
Technical Overview

Analysis of Peptide/Protein*-Related Impurities Using the Integrated Solution of Bio 2D-LC/Q-TOF in Agilent MassHunter Software

Suitable for Agilent
1290 Infinity III LC



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Abstract

This technical overview illustrates:

- The integration of the 2D-LC functionality into the Agilent MassHunter LC/MS software for the detection of high-resolution, accurate mass data from ¹D and ²D separations
- The practical use of the Bio 2D-LC/Q-TOF MS solution in the analysis of related impurities of a forced-degraded peptide/protein

The data demonstrate:

- The combination of 2D-LC with Q-TOF MS to be invaluable for the overall separation result
- The flexibility to vary the size of fractions sampled from ¹D, while substantial time savings can be achieved in ²D using Multi-Inject
- The compensation for retention time shifts in ¹D using dynamic peak parking
- A ²D-method development approach using the shifted gradient

Introduction

From contamination in food and concerns about pollutants in the environment, to the need to analyze complex samples in chemicals and the life sciences, there is a continually increasing demand for separation power.[1-5] Chromatography and mass spectrometry are powerful separation techniques individually, but their conjunction strongly amplifies the performance of the analytical platform. Two-dimensional liquid chromatography (2D-LC) enhances the separation power even further, because the second dimension (^{2}D) can improve the resolution of the separation achieved in the first dimension (^{1}D). This works best if selectivities of the two dimensions are orthogonal.[6]

2D-LC has progressed from being used predominantly in academic research, to being adopted by a wide community in industry. The drivers for this progression were the obtainability of commercially available systems and integrated software solutions specifically designed for 2D-LC workflows.[1]

Figure 1 shows the Agilent 1290 Infinity II Bio 2D-LC System**. In principle, it consists of two HPLC systems, one for each dimension. The autosampler of the second dimension (^{2}D) is replaced by the multiple heart-cutting (MHC) interface, incorporating an active solvent modulation (ASM) 2D-LC modulator and two MHC decks (A and B), in total holding 12 sampling loops.[4,7] The interface samples fractions from ^{1}D in these loops and online-injects these samples into ^{2}D.

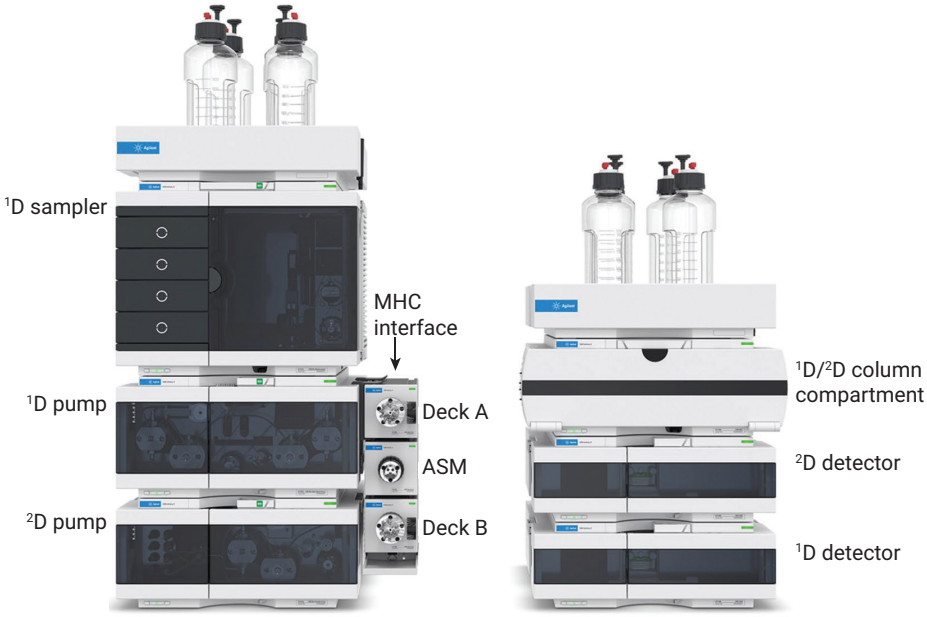

Figure 1. Agilent 1290 Infinity II Bio 2D-LC system equipped with Active Solvent Modulation 2D-LC modulator and Multiple Heart-Cutting decks.

The MHC interface can cover the complete range of 2D-LC modes. The comprehensive mode aims to produce a profile for all components present in a sample, and is typically applied to unknowns. No sample should be lost, which conditions a direct link of the ^{2}D cycle for each fraction taken from ^{1}D, and the rate at which sampling occurs. In comprehensive mode, only one loop of each MHC deck is used, and the 2D-LC modulator governs positioning for ^{1}D sampling and ^{2}D processing.[7] The purpose of multiple heart-cutting mode is different in that only target sections of a ^{1}D chromatogram are further examined. The link between time scales of the two dimensions is decoupled, because the MHC deck positioned in the ^{1}D path can store fractions, while the other deck is processed in ^{2}D. When this process is finished, the deck becomes ready to take further fractions, which extends the number of samples that can be examined in the second dimension.[3,7,9]

These workflows use sophisticated, 2D-LC-specific software solutions, incorporating clever algorithms to eliminate the operator burden to program myriads of valve switches and ^{2}D gradients. To synchronize these variables with the correct analysis time would truly be time-consuming and error-prone.

This technical overview illustrates the integration of the 2D-LC functionality into the Agilent MassHunter LC/MS workstation for detection of high-resolution, accurate mass data from ^{2}D separations using Q-TOF mass spectrometry. The practical use is demonstrated on the analysis of related impurities of a forced-degraded peptide/protein separated using MHC 2D-LC modes.

Overview of Agilent MassHunter 2D-LC software

Figure 2 shows a 2D-LC user interface of the Agilent MassHunter 11 Acquisition software. The Instrument Status (A) contains the 2D-LC control panel with the online monitor, providing event information during the course of a 2D-LC run. A deck colored in blue indicates its location in the ^{1}D path for sampling, and red means that the deck is processed in ^{2}D. The current situation shown is that of cut 1, taken from ^{1}D at 13.01 minutes, and is ^{2}D analyzed, while deck B is ready to take ^{1}D fractions.

The Method Editor (B) evolved in close collaboration with users in the industry, with a focus on simplifying the method setup for 2D-LC workflows. It is organized in three sections:

- Section B.1 is where operation mode, flow rates, types of solvents, and the stop time of 2D-LC runs are defined.
- Section B.2 contains tabular information on sampling, 2D-gradients, and settings for automated peak recognition (peak trigger).
- Section B.3 is the preview-centric section, allowing a visual 2D-method setup.

The preview shows a reference ^{1}D chromatogram, and the grey triangles on top of the chromatogram indicate where ^{1}D-peaks are recognized, which depends on the peak trigger (baseline threshold and/or slope). The highlights indicate where heart-cuts are predicted to be taken. Green highlights stand for time-based sampling, and blue for peak-based sampling.

In time-based mode, sampling from ^{1}D is triggered by time events as specified by the operator (i.e., in reference to the ^{1}D chromatogram). For instance, cut 1 at 13.01 minutes was generated by a double click on the grey triangle on top of the corresponding peak. The second time-based section is the high-resolution (HiRes) sampling series at 16 minutes. It predicts three cuts screening the main peak.

In peak-based mode, the decision to take fractions from ^{1}D is made "on-the-fly", where sampling occurs whenever the ^{1}D detector signal fulfills the criteria that were set in the peak trigger section. This can be useful if the appearance of the ^{1}D chromatogram is unknown, or in cases where retention in ^{1}D varies. The preview shows two peak-based regions. One defines a heart-cut at approximately 14 minutes, and the other defines a HiRes series of four cuts at

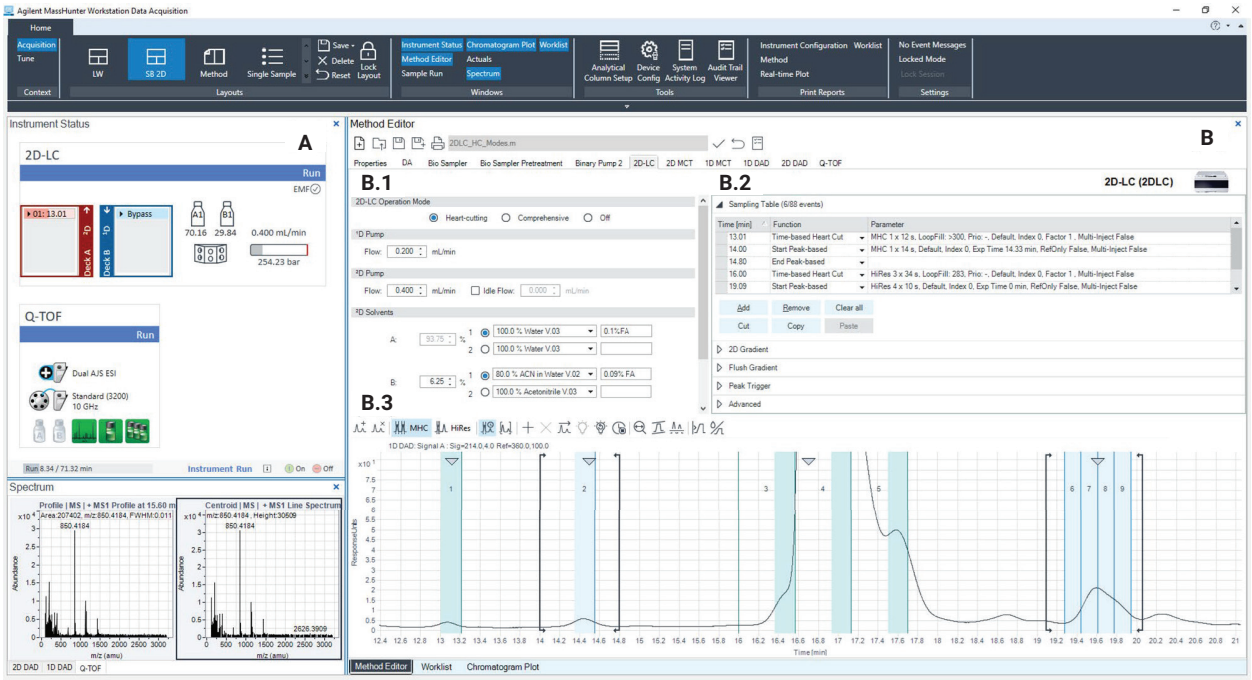

Figure 2. User interface of Agilent MassHunter Workstation LC/MS Data Acquisition with 2D-LC, version 11.0.

approximately 19 minutes. Peak-based regions are designated by brackets defining the start and end of peak-based operation, which can be moved to widen or shorten the interval.

The size of the highlight corresponds to the volume sampled from ^{1}D, meaning that for full loop sampling, the highlight equals the size of the loop (cuts 1 to 5, 40 µL) and for loop underfill experiments, the size is reduced (cuts 6 to 9, 33 µL).

All sections are synchronized, meaning that all information can also be gathered from the corresponding sampling table (compare B.2) and any change in one section (grabbing a highlight and moving it to a different position) is updated in the other sections (for example, in the sampling table).

Other method parameters can be visually set up in the preview in a similar fashion. This includes ^{2}D gradients, flush gradient, peak trigger, 2D-LC stoptime, and the optimization of the cut storage pattern in the MHC decks. More detailed insights are provided in the 2D-LC user manual.[10]

Experimental

Equipment

The Agilent 1290 Infinity II Bio 2D-LC/Q-TOF MS system:

First dimension (^{1}D):

- Agilent 1290 Infinity II Bio High-Speed Pump (G7132A)
- Agilent 1290 Infinity II Bio Multisampler (G7137A)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) equipped with a bio-inert standard flow heat exchanger (G7116-60071)
- Agilent 1290 Infinity II Diode Array Detector (G7117B) with Max-Light Cartridge Cell LSS (10 mm) (G7117-60020)***

Second dimension (^{2}D):

- Agilent 1290 Infinity II Bio High-Speed Pump (G7132A)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) equipped with a bio-inert standard flow heat exchanger (G7116-60071)
- Agilent 1290 Infinity II Diode Array Detector (G7117B) with Agilent Max-Light Cartridge Cell LSS (10 mm) (G7117-60020)***

2D-LC interface

- Agilent Infinity Lab Bio 2D-LC ASM Valve (2D-LC valve with active solvent modulation, G5643B) including two Agilent InfinityLab MHC Valves with biocompatible sample loops
- 3× Agilent 1290 Infinity II Valve Drives (G1170A)
- Agilent 2D-LC Pressure Release Kit (G4236-60010)

The Agilent 6545 LC/Q-TOF equipped with Dual AJS ESI acquired MS data in both dimensions (G6546A).

Software

- Agilent MassHunter Workstation LC/MS Data Acquisition, Version 11.0 with 2D-LC Software (G2198AA#410)
- Agilent MassHunter Worstation Qualitative Analysis, Version 10.0
- Agilent MassHunter Workstation Software BioConfirm, Version 10.0

Columns

- **^{1}D:** Agilent InfinityLab Poroshell HPH, 150 × 2.1 mm, 2.7 µm (part number 693775-702)
- **^{2}D:** Agilent InfinityLab Poroshell HPH, 50 × 2.1 mm, 2.7 µm (part number 699775-702)

Chemicals

All solvents were LC grade. Acetonitrile (ACN) was 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, Merck-Millipore, Billerica, MA, USA). Trifluoro acetic acid (TFA) and formic acid (FA) were obtained from Sigma-Aldrich (Steinheim, Germany).

Samples

Samples used in the study were generated by exposing 1 mg/mL of a peptide/protein model compound (bovine insulin from Sigma-Aldrich, St. Louis, MO) in 25 mM of sodium phosphate, pH 7 at 50 °C for 5 days.

Method

Table 1. Chromatographic conditions.

Parameter	Value
First Dimension (^{1}D)	
Solvent	A) 0.1% TFA in water B) 0.09% TFA in ACN: water 80:20 v:v
Gradient	Time (min) %B 0 31 60 40 61 80 62 80 63 31
Flow Rate	0.300 mL/min
Temperature	40 °C
Detection	214 nm 40 Hz
Injection	Injection volume: 5 µL Needle wash: 3 s in 0.1% FA in ACN/water 50/50 v/v

Parameter	Value
Second Dimension (^{2}D)	
Solvent	A) 0.1% FA in water B) 0.09% FA in ACN/water 80/20 v/v
Gradient	Time (min) %B 0 6.25 0.01 25 2.3 34 2.4 90 2.5 90 Analysis: 2.5 min Equilibration: 1.6 min Cycle time: 5.0 min
2D-LC Modulator	ASM valve operated with ASM-f 3
Flow Rate	0.400 mL/min
Temperature	40 °C
Detection	214 nm 40 Hz
Injection	Sampling loop: 40 µL

Parameter	Value
LC/Q-TOF	
Polarity	Positive
Dual AJS ESI Source	Gas temperature: 320 °C Drying gas: 8 L/min Nebulizer: 35 psi Sheath gas temperature: 350 °C Sheath gas flow: 11 L/min VCap: 3,500 V Nozzle: 500 V
MS TOF	Fragmentor: 175 V Skimmer: 65 V Instrument mode: 10 GHz Mass range: 100 to 3,500 *m/z* Acquisition rate: 5 spectra / s Reference mass: 121.0509 and *m/z* 922.0098

Results and discussion

HiRes/peak-based operation

A new option for 2D-LC in MassHunter is HiRes operation, in conjunction with peak-based sampling, which was used to provide further insight into the main peak.

Figure 3A shows an excerpt of the reference ^{1}D chromatogram, with nine cuts of the HiRes series sampling nearly the entire band for reprocessing in ^{2}D. Panel B shows ^{2}D results for cuts 1, 2, 4, 6, and 9, in the form of extracted ion chromatograms (EICs) obtained by summing isotopic patterns of charge states +3 to +6. Panel C shows the spectra of charge state +4 for the bovine insulin, with a corresponding de-amide superimposed.

The ^{1}D results in panel A suggest at least three components under the main peak, with shoulders in the front and rear. This was confirmed by deconvoluted MS data

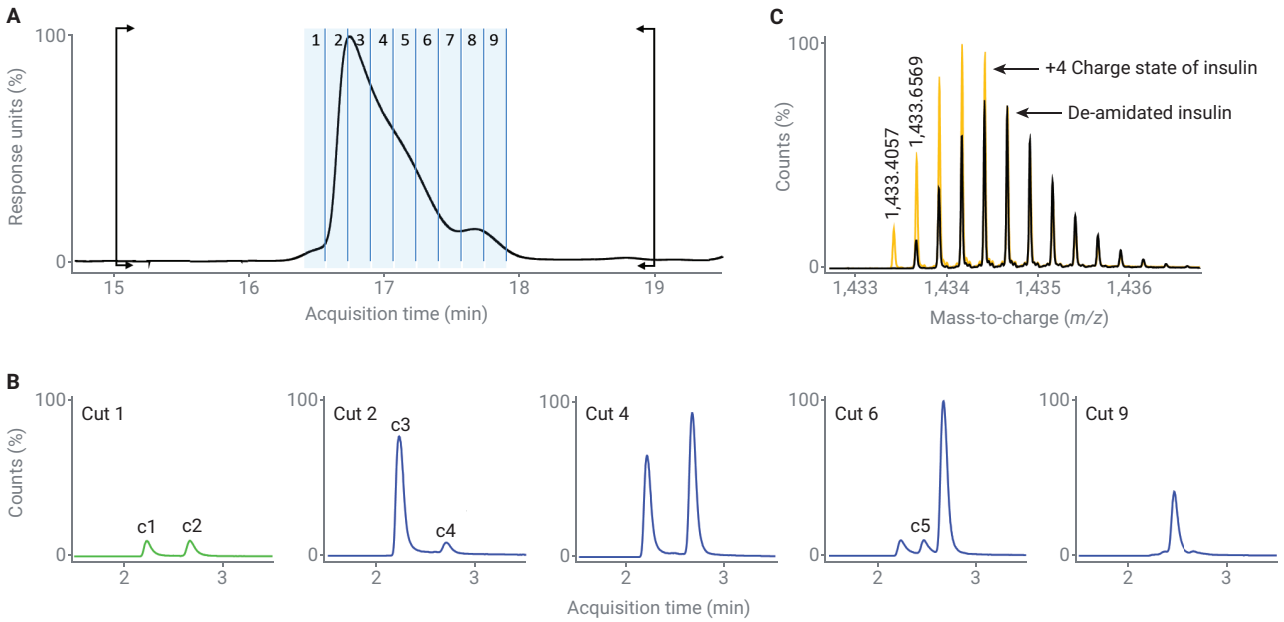

Figure 3. (A) ^{1}D chromatogram reference chromatogram with overlaid highlights for the HiRes series. (B) ^{2}D results in the form of extracted ion chromatograms (EIC). (C) MS spectra of charge state +4 for insulin, and deamidated insulin.

(BioConfirm: max. entropy algorithm) providing monoisotopic masses of 5,729.6 Da (at apex of the main peak), 5,730.6 Da (at the rear), and 5,658.6 Da (at the front), which could be assigned to the intact bovine insulin, a deamidation product, and a loss of alanine at the C-terminus of the b-chain, respectively.

In ^{2}D, five compounds (c1 to c5 in panel B) were detected. c1 was due to the alanine loss, c2 a corresponding de-amide, c3 the intact insulin, and c4 and c5 two de-amides of insulin. This demonstrates the importance of the chromatographic separation. Isomers cannot be distinguished by mass spectrometry, and it is also not trivial to discriminate the intact molecules from corresponding de-amides when entering the MS at the same time (i.e., when coelution in LC occurs) because the second isotope of the intact mass approximately coincides with the monoisotopic mass of the de-amide, as can be gathered from panel C. On the other hand, c1 and c3, and c2 and c4 would have coeluted also in the second dimension (there was a low abundance of c1 and c2 also in cut 2). Yet, Q-TOF data allowed separation by extracting corresponding masses.

Thus, the combination of both separation techniques (2D-LC and Q-TOF) has been demonstrated to be invaluable for the overall result.

HiRes/peak-based combined with Multi-Inject operation

The Multi-Inject function is an option that works in conjunction with the HiRes sampling mode. Multi-Inject can be useful to save time in 2D-LC experiments, and allows flexibly setting the sampling volume through software settings, without the need for hardware modifications.

Figure 4 shows the continuous UV trace of ^{2}D chromatograms, along with ^{2}D cut markers (purple trace) obtained for the series of nine cuts taken from ^{1}D as shown in Figure 3A. ^{2}D cut markers can be obtained from data analysis using MassHunter Qualitative Analysis software to indicate where cuts were analyzed in relation to the ^{1}D time scale. Panel A is from normal HiRes operation, and panel B was obtained when Multi-Inject was activated.

In HiRes mode, the second dimension runs through nine consecutive ^{2}D cycles. ^{2}D cut markers in panel A show that ^{2}D processing finished at approximately 68 minutes.

In Multi-Inject mode, the content of each MHC deck (i.e., five loops in deck A and four loops in deck B) is first transferred to the ^{2}D column (marked in panel B with TC (transfer-to-column)) before the entire deck is analyzed by a single ^{2}D cycle. The instrumental setup used 40 µL sampling loops. Thus, Multi-Inject could be viewed as replacing these by a single 200 µL loop (deck A) and a 160 µL loop (deck B) but without replumbing the hardware. ^{2}D cut markers show that the ^{2}D analysis time was markedly reduced from 68 to 39 minutes.

From a performance point of view, it is important that the compounds analyzed concentrate on the ^{2}D column head. To support this, ASM with an ASM-f 3 (dilution factor 3) was used in all experiments. ASM was thoroughly described in the literature.[13] Briefly, ASM is a valve-based approach to dilute the loop content with weak solvent prior to its arrival at the ^{2}D column. The weak solvent is delivered by the ^{2}D pump at the start of each ^{2}D cycle during the sample transfer period (here, 6.25 %B). The software automatically switches the ASM valve into an intermediate position for dilution, and once the sample in the loop has been displaced, into the final ^{2}D analysis position.

Figure 4C shows an overlay of cuts 1 to 5 and 4 obtained from and Multi-Inject (green trace) and HiRes (blue trace), respectively, and correspondingly, cuts 6 to 9 and 7.

Similar peak shapes were obtained in both experiments, with a maximum increase in peak width (FWHM) of approximately 10% in Multi-Inject compared to HiRes, which for these chromatograms had little influence on the resolution. Excellent agreement was obtained with respect to peak area. For Multi-Inject, compared to HiRes, 99.1, 100.9, and 100.8% were calculated for peaks c3, c5, and c4, respectively (in HiRes, summing of all areas is obtained per compound).

Multi-Inject successfully reduced the ^{2}D analysis time by approximately 30 minutes. The time saving can become even more significant for longer ^{2}D cycle times. It is not unusual to use more than 30 minutes per ^{2}D cycle in experiments aiming for the separation of isomeric compounds of peptide/protein-related impurities. In addition, Multi-Inject introduces flexibility, to vary the size of fractions sampled from ^{1}D.

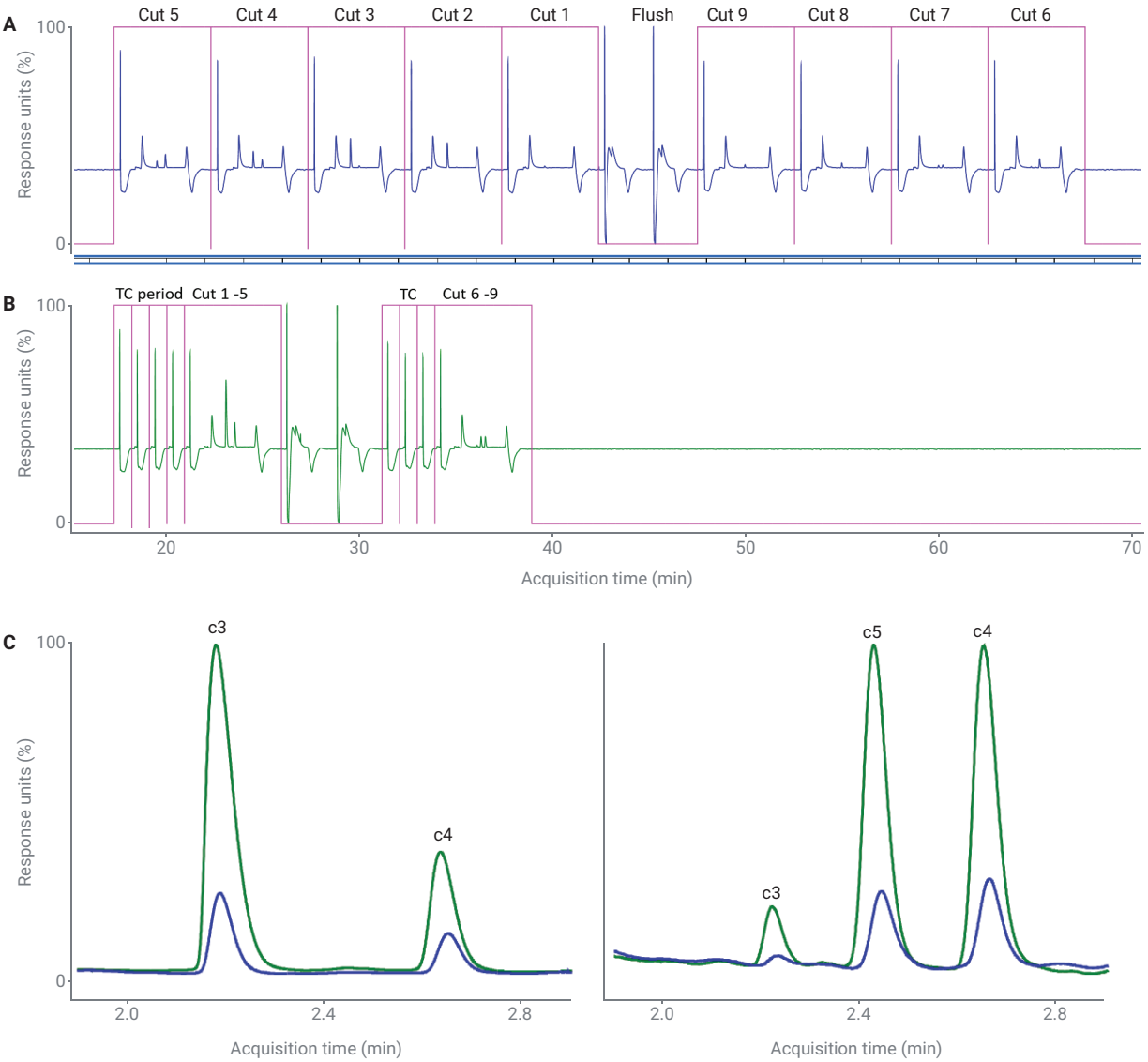

Figure 4. Continuous UV-trace of ^{2}D chromatograms (blue) along with ^{2}D cut markers (purple). Cut numbers were added to indicate related chromatogram sections. (A) Data obtained from peak-based HiRes mode. (B) Data obtained from peak-based HiRes mode with Multi-Inject activated. (C) Overlay of ^{2}D results (UV) for cuts 1 to 5 (Multi-Inject) and 4 (HiRes; left), and correspondingly cuts 6 to 9 and 7 (right).

Dynamic peak parking

In MHC 2D-LC, where cuts are taken in reference to a ^{1}D chromatogram, a fluctuation in retention results in a moving target, which can pose a marked challenge on the sampling of ^{1}D peaks. This is a general problem with large molecules, especially in combination with very shallow gradients required to separate related impurities of peptides (here: 0.15 %B/minute at 0.3 mL/minute). Peak-based operation can compensate for ^{1}D retention shifts, but is often not viable for the analysis of peptide impurities due to high sample complexity and poor separation.[14]

Dynamic peak parking (DPP) addresses the moving target issue in time-based sampling mode.

Figure 5A shows an excerpt of the preview of MassHunter with a ^{1}D chromatogram, with reference to which seven time-based heart-cuts (cuts 2 to 8) were defined. Cut 1 is sampled in peak-based mode, and serves as internal retention time standard (IRTS), which is required for DPP. Cuts 2 to 8 are indexed to this IRTS. An IRTS should exhibit the same (or at least very similar) retention-time properties to those heart-cuts linked to it. Thus, with DPP activated, a move in retention time of the IRTS is transmitted to the time-based cuts dynamically, whose sampling subsequently shifts in real time by the same or a scaled time difference.

This is demonstrated in Figure 5B, showing ^{1}D chromatograms along with ^{1}D cut markers (purple). ^{1}D cut markers are obtained from data analysis using MassHunter Qualitative Analysis Software, and indicate where cuts were sampled from the ^{1}D chromatogram. These chromatograms were acquired on a different date than those in panel A. Panel B.1 (green) was the first run of the day, and panel B.2 (orange) a repetition.

Both chromatograms in panel B (in comparison to panel A) show a shift to somewhat earlier retention times, and each to a different extent. DPP has compensated for these shifts, as indicated by the ^{1}D cut markers. Without this compensation, time-based cut definitions made in panel A would have been missed.

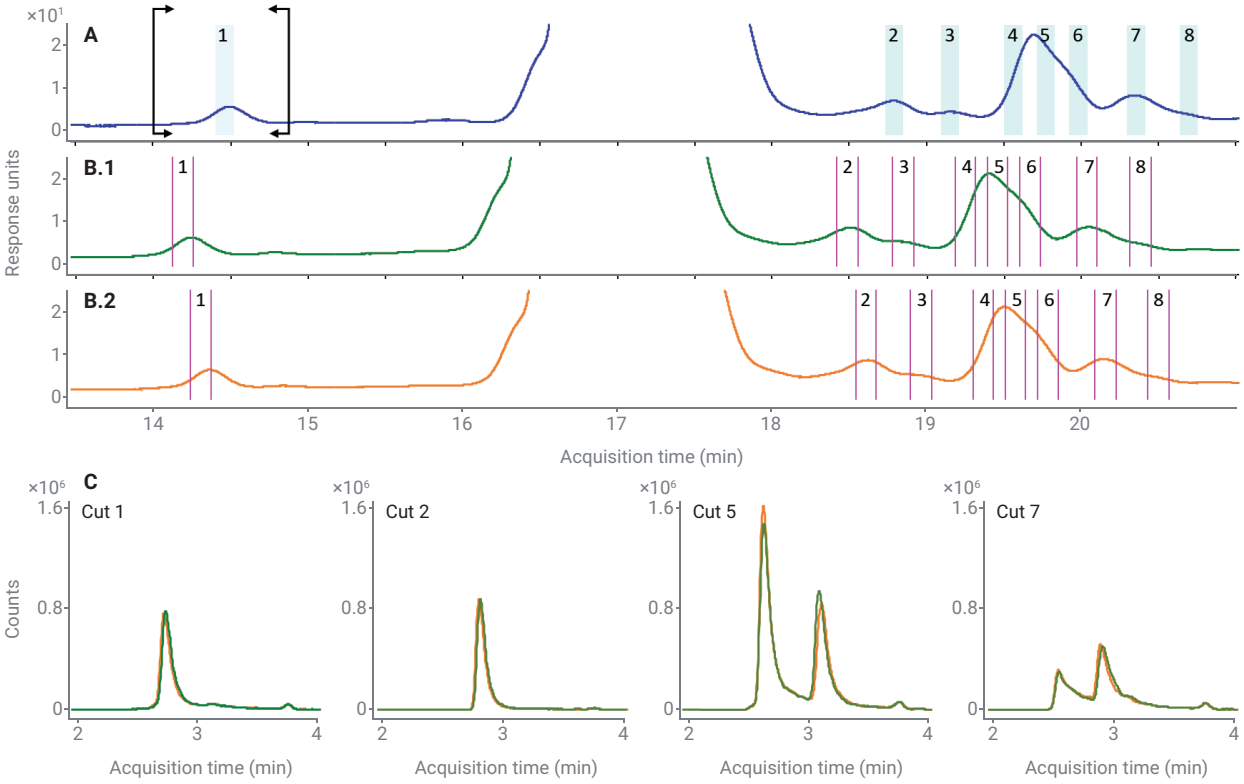

Figure 5. (A) ^{1}D chromatogram from preview of Agilent MassHunter Acquisition Software with IRTS (cut 1) defined by peak-based interval and time-based cuts (2 to 8) indexed to the IRTS. (B.1) A ^{1}D chromatogram (green) and ^{1}D cut markers (purple) obtained from Agilent MassHunter Qualitative Software. (B.2) A repetition of B.1, the ^{1}D chromatogram (orange) and cut markers (purple). (C) ^{2}D extracted ion chromatograms obtained from analysis in B.1 and B.2 superimposed, corresponding color shown as in B.

Figure 5C shows an example of ^{2}D results for cuts 1, 2, 5, and 7, obtained from ^{1}D chromatograms in panels B.1 and B.2, superimposed. Near-congruency confirms that retention variations in ^{1}D were well compensated for by the DPP operation.

Shifted Gradient for method development in ^{2}D

The shifted gradient is used to modify the solvent composition in ^{2}D gradients across the 2D-LC run, and can therefore be useful for method development and optimization of the second dimension. MHC 2D-LC is a convenient tool for this purpose, because several small fractions can be taken across coeluting regions in ^{1}D.

This is shown in Figure 6A, where four heart-cuts in each of the two ^{1}D regions (1 and 2) are defined. The shifted gradient is indicated by the black dotted line, connecting the bullet setpoints. Setpoints can be generated in the preview, and maneuvered to modify the solvent composition of the ^{2}D gradient. The resulting shifts are taken up by the table section of the Method Editor. The initial ^{2}D gradient in this experiment had its starting composition at 26 %B, and its end composition at 30 %B. It is possible to shift both the start- and end-condition of the ^{2}D gradient. For reasons of simplicity, the results shown in Figure 6 were generated shifting the upper %B level only.

Panel A can be interpreted as follows: The first setpoint of the shifted gradient was set to 16.33 minutes, which is the time where sampling of cut 1 starts (the left edge of the blue highlight). The black dotted line up to this point is flat, and therefore, no ^{2}D gradient shift occurs. Since the ^{2}D gradient is linked to the start of the fraction sampled, cut 1 will be analyzed using the initial gradient from 26 to 30 %B. The second setpoint was at 40 %B at the start of cut 4, which is therefore analyzed using the range of 26 to 40 %B. For cuts 2 and 3, the %B level is determined from where the line connecting the two setpoints crosses the start of each corresponding highlight.

In a similar fashion, cuts 5 to 8 were analyzed, with 40 %B for cut 5, 30 %B for cut 8, and intermediate values for cuts 6 and 7. Corresponding gradient traces are shown in Figure 6B, and panel C shows ^{2}D EIC chromatograms. These were processed in data analysis to be displayed relative to the ^{1}D time scale (i.e., where they were analyzed in real time). Based on earlier results (Figure 3), three compounds c3 to c5 were expected. Cut 5 in Figure 6C shows compounds c3 and c4 to closely elute. It is likely that c5 coelutes with one of these components. Cut 8 shows strongly

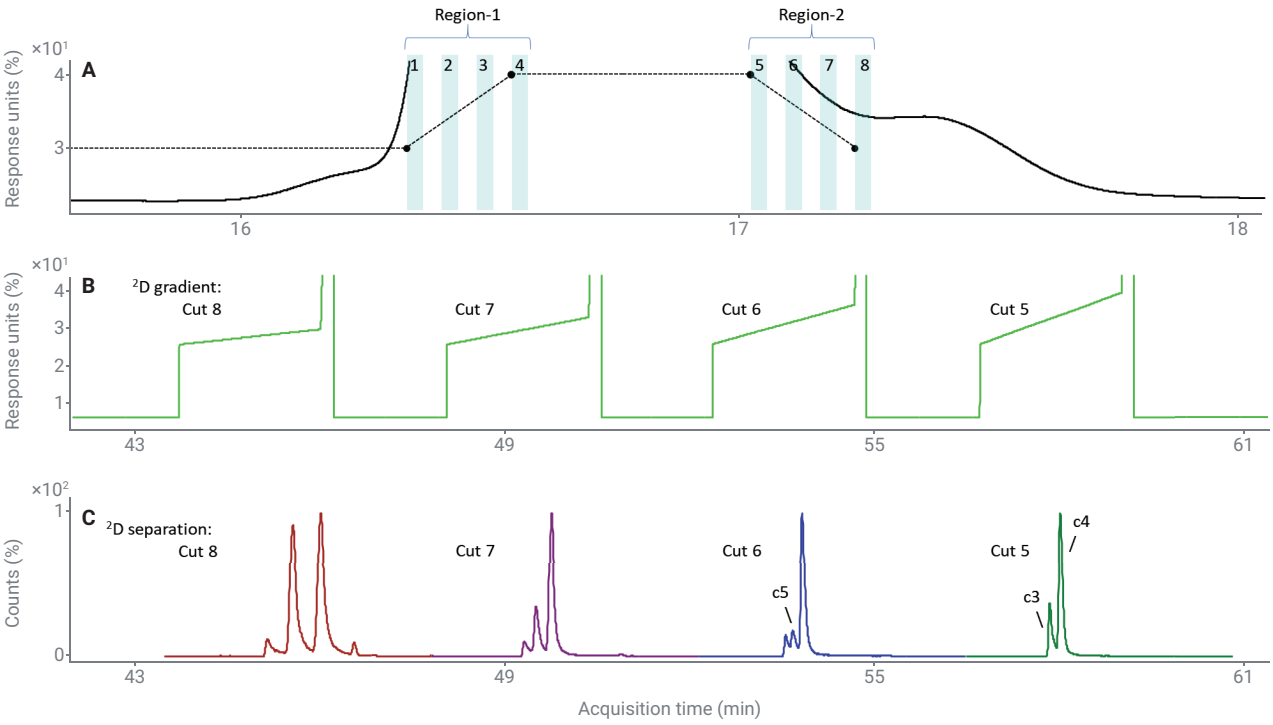

Figure 6. (A) ^{1}D chromatogram from a preview of Agilent MassHunter Acquisition software time-based MHCs and shifted gradient. (B) ^{2}D shifted gradient traces for cuts 5 to 8 obtained from Agilent MassHunter Qualitative software. (C) ^{2}D chromatograms obtained from the analysis in A and B, extracted using the Keep ^{1}D Retention Time function of the MassHunter Acquisition software.

improved separation, with all compounds being well-separated. However, peaks were relatively broad, and in addition, part of c4 was affected by the flushing step of the ^{2}D gradient (90 %B at 2.4 minutes, see the Experimental section) so that an apparent fourth compound appeared. This was, however, an artifact. Cut 7 gave the best compromise between resolution and peak width, and thus, the corresponding ^{2}D gradient condition was chosen for further optimization, which included determination of the ^{2}D gradient starting condition, and a fine-tune of %B at start and end using the same shifted gradient approach.

Note:

- If ^{1}D peaks become narrow, use smaller sampling loops. In this study, 10 µL loops were used (as per definition in the sampling loop configuration).
- If MHC cuts are too close to each other, cuts may be lost. In this study, the distance of cuts was 0.07 minutes (cuts 5, 6, 7, and 8 were taken at 17.02, 17.09, 17.16, and 17.23 minutes, respectively).
- Sample cuts of a ^{1}D region in one parking deck (cuts 1 to 4 in Figure 6A are considered to belong to region 1). In this study, this was forced in the sampling table by setting the ^{2}D analysis mode of cuts 1 to 3 and 5 to 7 to delayed, and cuts 4 and 8 to being analyzed in default mode, which led to the storage of cuts 1 to 4 in deck A and cuts 5 to 8 in deck B.

Technically, such scouting experiments could be run in 1D-LC and results translated to ^{2}D. However, compared to this approach, optimizing the method in ^{2}D has advantages. First, there is no need to correct for parameters related to the system configuration (gradient dwell volume/time), but results can be directly applied to the actual 2D-LC experiment. Second, for complex samples such as those used here, MHC markedly reduces the complexity by taking fractions from ^{1}D, which then become the new sample for ^{2}D. In this way, method optimization is markedly simplified.

Conclusion

The use of a Bio 2D-LC/Q-TOF MS was illustrated on the analysis of related impurities of a peptide/protein. The hyphenation of both separation techniques (2D-LC and MS) provided the most comprehensive information on peak purity. The key for operation of this analytical platform is software which incorporates clever algorithms for intuitive visual programming of 2D-LC experiments.

The new features of the Agilent MassHunter software for 2D-LC, including Multi-Inject, dynamic peak parking, and an update on shifted gradient were demonstrated to have enabled:

- Flexible variation of ^{1}D sample size
- Marked time-savings in ^{2}D analysis time
- Compensation of the moving ^{1}D target issue
- Method development and optimization in 2D-LC

Footnotes

* **Peptide/protein:** Insulin has a tertiary structure and consists of two peptide chains, so technically it is a small protein. Chromatographically, it behaves more like a peptide. Proteins are typically large (>10 kDa), and produce poor reversed-phase chromatograms with few broad bands. In contrast, peptides give complex chromatograms with relatively narrow peaks.

** Configurations of a Bio 2D-LC and standard 2D-LC solution are identical. Bio 2D-LC uses biocompatible modules and contains parts produced from MP35N alloy, PEEK, or ceramics, which are known to increase the tolerance towards harsh mobile phases (e.g., high salt content) and to reduce the interaction of internal surfaces with bio molecules.[8]

*** **Note:** High-sensitivity detection can cause photo-oxidation of biomolecules.[11] The Agilent Max-Light Cartridge Cell LSS (G7117-60020) has an aperture at the inlet, which decreases the energy intake and thus can reduce light-induced degradation without marked influence on the signal-to-noise ratio.[12] The possibility that this measure of safety (LSS) was not sufficient, was tested in an experiment where UV lamps were switched off. For data reported here, no marked difference was observed with or without the diode array detector. For extremely vulnerable compounds, alternatives are switching off the light (if possible) or the use of detectors with lower light intensity (e.g., single wavelength detectors).

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