Complementary analysis in a biopharmaceutical research laboratory with a 2D-LC-MS instrument for flexible operation

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Application benefits

- Multi heart-cut 2D-LC-MS provides an efficient approach for impurity characterization of a therapeutic protein.
- Thermo Scientific™ Simple Switch™ technology enables instrument use in multi heart-cut 2D-LC-MS, 1D-LC-MS, or Dual 1D-LC mode for the most flexible equipment and lab space utilization and maximum MS productivity.
- 2D-LC-MS and 1D-LC-MS experiments for top-down and bottom-up analysis of the proteins can be implemented with the same instrument without any hardware changes.

Goal

Identity confirmation of protein-based impurities of a therapeutic protein by multi heart-cut 2D-LC-MS in a top-down experiment.

Introduction

Two-dimensional liquid chromatography (2D-LC) implies the transfer of eluate fractions of one LC separation (first dimension, ^{1}D) into another LC separation step (second dimension, ^{2}D) to improve the resolution of highly complex samples or difficult to resolve mixtures. The scope ranges from single fraction transfer (heart-cut 2D-LC) via several

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fractions (multiple heart-cut 2D-LC) through comprehensive 2D-LC mode when the complete ^{1}D eluate is cut into small equal portions and transferred into the ^{2}D. The automated fraction transfer in online 2D-LC techniques is facilitated by various interface designs, usually consisting of one or several switching valves and storage loops or traps.[1] 2D-LC application fields cover all important areas in analytical chemistry, such as pharma, biopharmaceuticals, food and beverage, natural medicines, proteomics, metabolomics, and industrial chemicals, where 2D-LC can be utilized for tasks from simple, like desalting for mass spectrometry, to complex, with maximized peak capacity for the highest resolution in multi-component analyses.[2, 3]

In the recent decade, 2D-LC has gradually transformed from an advanced "specialists only" technique into common practice, mainly driven by the commercial availability and support from LC equipment manufacturers. Although 2D-LC instrumentation has found its way into a variety of labs, its usage might still not be an everyday routine in each of them. Frequently, either dedicated 2D-LC systems stand idle waiting for the next 2D-LC application to come, or have to be re-plumbed for 1D-LC usage to increase efficiency in terms of lab space utilization and the return on investment (ROI). The latter is especially true when a valuable mass spectrometer (MS) is connected to the system.[3]

This application note demonstrates how the Simple Switch™ technology in a Thermo Scientific™ Vanquish™ Horizon Online 2D-LC system provides a convenient solution to users in such situations to get the most out of their system. In a biopharmaceutical R&D laboratory, a multiple loop heart-cut 2D-LC-MS system is used for the impurity characterization of a therapeutic protein. Moreover, by incorporating a Thermo Scientific™ Vanquish™ Dual Split Sampler into the system, the ^{2}D flow path is equipped with an independent injection unit, enabling direct injection and 1D-LC-MS in that flow path when the 2D-LC-MS functionality is not required.

Experimental

Chemicals

Reagent	Grade / purity	Cat. no.	Supplier
Trifluoroacetic acid (TFA)	Sequencing grade	28904	Thermo Fisher Scientific
Formic acid (FA)	UPLC/MS	069141	Biosolve
Acetonitrile (mobile phase only)	UPLC-MS	012041	Biosolve
Water (mobile phase only)	UPLC-MS	232141	Biosolve

Sample handling

Item	Description	Cat. no.	Supplier
Sample preparation tubes	Eppendorf™ Safe-Lock™ Tubes, 1.5 mL	0030120086	Eppendorf
Screw neck vials with caps UHPLC	Polypropylene, 12 × 32 mm, 0.3 mL volume	186002639	Waters Corporation

Sample preparation

The protein sample was dissolved in a 25 mM ammonium bicarbonate buffer (pH 8) and reduced by dithiothreitol (DTT) following a standard procedure to facilitate top-down fragmentation and increase sequence coverage.

Instrumentation

A Thermo Scientific™ Vanquish™ Horizon Simple Switch™ 2D-LC system for multiple loop heart-cutting was used, enabling either the collection of up to five fractions from a ^{1}D separation and the transfer to a ^{2}D separation, or alternatively allowing the operation of two standard 1D-LC separations in the dual fluidic paths. The system consisted of:

- System Base Vanquish Flex/Horizon (P/N VH-S01-A-02)
- Vanquish Stack Stabilizer (P/N 6036.1710)
- 2× Vanquish Binary Pump H (P/N VH-P10-A-02)
- Vanquish Dual Split Sampler HT (P/N VH-A40-A-02)
- 2× Vanquish Column Compartment H (P/N VH-C10-A-02)
- Valve 2-position/6-port 150 MPa bio (P/N 6036.1560)
- 2× Valve 6-position/7-port 150 MPa bio (P/N 6036.1570)
- 3× Loop, MP35N, 50 µL (P/N 6823.0029)
- 3× Loop, MP35N, 250 µL (P/N 6823.0030)
- 2× Vanquish Diode Array Detector FG (P/N VF-D11-A-01)
- 2× Flow Cell, Standard Bio, 10 mm, 13 µL (P/N 6083.0540)
- Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer (P/N IQLAAEGAAPFADBMBCX)

Chromatographic conditions

Table 1. Chromatographic conditions of ^{1}D

Parameter	Value
Column	Waters™ Acquity™ UPLC Peptide BEH C18, 2.1 × 50 mm, 1.7 µm, 300 Å
Mobile phase	A: 0.05% TFA in water B: 0.04% TFA in acetonitrile
Flow rate	0.6 mL/min
Injection volume	8.4 µL
Gradient	*Time (min)* *%B* 0.0 50 3.0 50 8.0 70 8.1 90 11.0 90 11.1 50 18.0 50
Column temperature	40 °C (still air incl. active eluent preheating and post-column cooling)
Autosampler temperature	5 °C
UV wavelength	215 nm, 280 nm
UV data collection rate	5 Hz
UV response time	1 s
UV 3D field	190–310 nm, 4 nm bunch width
Inject wash	Before and after injection

Table 2. Chromatographic conditions of ^{2}D

Parameter	Value
Column	Tosoh™ UP-SW2000, 4.6 × 150 mm, 2.0 µm, 125 Å
Mobile phase	A: 0.1% FA in water B: 0.1% FA in acetonitrile 35% B (isocratic)
Flow rate	0.35 mL/min
Run time	10 min
Column temperature	25 °C (forced air)
Autosampler temperature	5 °C

While ^{2}D runs were performed, the ^{1}D was kept at the starting conditions and vice versa. In addition, a conditioning pre-run method was used to flush all loops to the starting conditions of the ^{1}D.

MS settings

For top-down data acquisition, a targeted MS/MS method was applied with either HCD, CID, or ETD fragmentation in separate consecutive runs applying a wide isolation window of 600 *m/z* to capture several charge states of the charge envelope. The 2D-LC runs were repeated to analyze the fractions consecutively by the different MS fragmentation methods.

Table 3. Instrument and scan settings for the mass spectrometer

Parameter	Value
Vaporizer temperature	300 °C
Ion transfer tube temperature	300 °C
Ion source voltage	+4,000 V
Sheath gas	80
Aux gas	19
Sweep gas	2
Targeted scan settings	
MS^{n} level	2
Isolation mode	Quadrupole
Isolation window	600 *m/z*
Detector type	Orbitrap
Resolution	60,000
Microscans	20
Activation types	HCD, CID, ETD

The LC effluent flow into the MS source was regulated by the internal diverter valve, directed to the ion source from 0 to 4.5 min and to waste afterwards to divert TFA.

Data acquisition and analysis

Thermo Scientific™ Chromeleon™ CDS software, version 7.2.9 was used for LC control and LC-UV data acquisition and analysis. Thermo Scientific™ Xcalibur™ 4.3 software was used for MS data acquisition. Thermo Scientific™ BioPharma Finder™ 4.1 software was used for MS data analysis. Thermo Scientific™ SII for Xcalibur 1.5 was used when the system was run in 1D-LC-MS mode.

Results and discussion

Online multi heart-cut 2D-LC-MS in a biopharmaceutical research lab

Biopharmaceuticals include protein- or nucleic acid-based macromolecular products, such as monoclonal antibodies (mAbs), antibody drug conjugates (ADCs), and RNA- or DNA-based therapeutics. Their analytical

characterization is usually challenging due to their size and heterogeneity making 2D-LC a valuable tool to tackle emerging challenges.[2] Favored separation modes comprise reversed-phase (RP) LC, ion-pairing RP-LC, hydrophobic-interaction, ion-exchange (IEX), and size-exclusion (SEC) chromatography, often coupled to MS detection.[1,2]

The objective of the 2D-LC-MS application was the confirmation of protein-based impurities of a proprietary therapeutic protein with an approximate molecular weight of 25 kDa applying top-down MS experiments. The identification of the main compound and several impurities was accomplished by a preceding separate bottom-up peptide mapping analysis not shown here. 2D-LC was utilized for separation followed by mass spectrometric confirmation and assignment of individual impurities in the obtained chromatogram.

The reduced protein sample was analyzed without further clean-up by the multiple loop heart-cut 2D-LC-MS system depicted in Figure 1. An RP-LC separation was used in the ^{1}D, separating the main peak and two unknown impurities, which were to be identified by MS fragmentation analysis. The ^{1}D gradient utilized mobile phases with TFA as modifier. TFA is a common modifier in LC and only occasionally used in LC-MS since it causes signal suppression for basic compounds analyzed by electrospray ionization mass spectrometry (ESI-MS) and in addition can cause corrosion of metal parts in the ion source. Attempts to substitute TFA with FA in the RP separation to enable sensitive 1D-LC-MS resulted in strong tailing of the main peak and made the impurity peaks disappear in the tail. To keep the resolution obtained with TFA but prevent TFA from affecting the MS sensitivity, a ^{2}D was used as a TFA removal step. An isocratic SEC separation with FA as modifier was set up in the ^{2}D. Each impurity peak was transferred into one of the 50 µL loops and stored by switching the 6-p-7-p selection valves. Heart-cut fractions were 0.08 min long, corresponding to 48 µL volume at the ^{1}D flow rate of 0.6 mL/min. In general, up to five fractions can be stored with the system; the sixth position serves as a bypass. After the ^{1}D run was finished, the loops were successively analyzed by the SEC method connected to the MS, where the target compounds eluted in single peaks into the MS, while late eluting small molecules like TFA were diverted to waste at the end of each ^{2}D run. The ^{2}D utilized MS detection only, without the optional UV detector being in line. Figure 1A shows the valve positions of the system during the ^{1}D for the collection of a fraction in loop 4 (2-p-6-p valve in position 1_2; both 6-p-7-p valves in position 4) as an example. When no fraction is collected, both 6-p-7-p valves are switched to the bypass position 6. Figure 1B depicts the valve configuration during the ^{2}D run of loop 4 (2-p-6-p valve in position 6_1; both 6-p-7-p valves in position 4) as an example.

An overlay of ^{1}D UV chromatograms of eight consecutive injections is shown in Figure 2. For each injection, 28.5 µg of sample were loaded onto the ^{1}D column. The separation was very robust and reproducible. The MS fragmentation of the separated protein-based impurities contained in the collected fractions in the ^{2}D resulted in a sequence coverage of 55% (first fraction) and 45% (second fraction), respectively, providing good sequence coverage, verification of postulated sites of modification, and confirmation of the results obtained from the preceding peptide mapping analysis.

Vanquish Online 2D-LC Systems are most easily controlled by Chromeleon CDS software as it provides features for most flexible instrument method setup, in particular through the definition of custom variables and availability of triggers for instrument control. These enable flexible heart-cut valve switches without the need to create a dedicated instrument method when switching times change. For multi heart-cut 2D-LC, it is most convenient to set up two instrument methods – one for ^{1}D and one for ^{2}D. These contain the triggers and references to custom variables, which are added to the sequence table. An example is shown in Figure 3 where the custom columns “FxStart”, “FxEnd”, and “LoopD2” in the sequence enable flexible setting of fraction starts and stops and the loop to be analyzed. ^{2}D runs are entered as sample type “Blank” into the sequence, which causes the autosampler to omit the injection procedure, to disable any autosampler action when loop content is transferred to the ^{2}D column. Template instrument methods and sequences containing the corresponding custom variables and triggers are available online with this Application Note in the Thermo Scientific™ AppsLab™ Library of Analytical Applications (https://appslab.thermofisher.com/). Control of the 2D-LC-MS instrument is also possible by SII for Xcalibur. Here, we chose to separately control the MS system via Xcalibur software and the 2D-LC by Chromeleon CDS to benefit from the full flexibility provided by the features explained above.[3,4] Communication between the two systems was achieved via respective state signals set in the MS and LC methods.

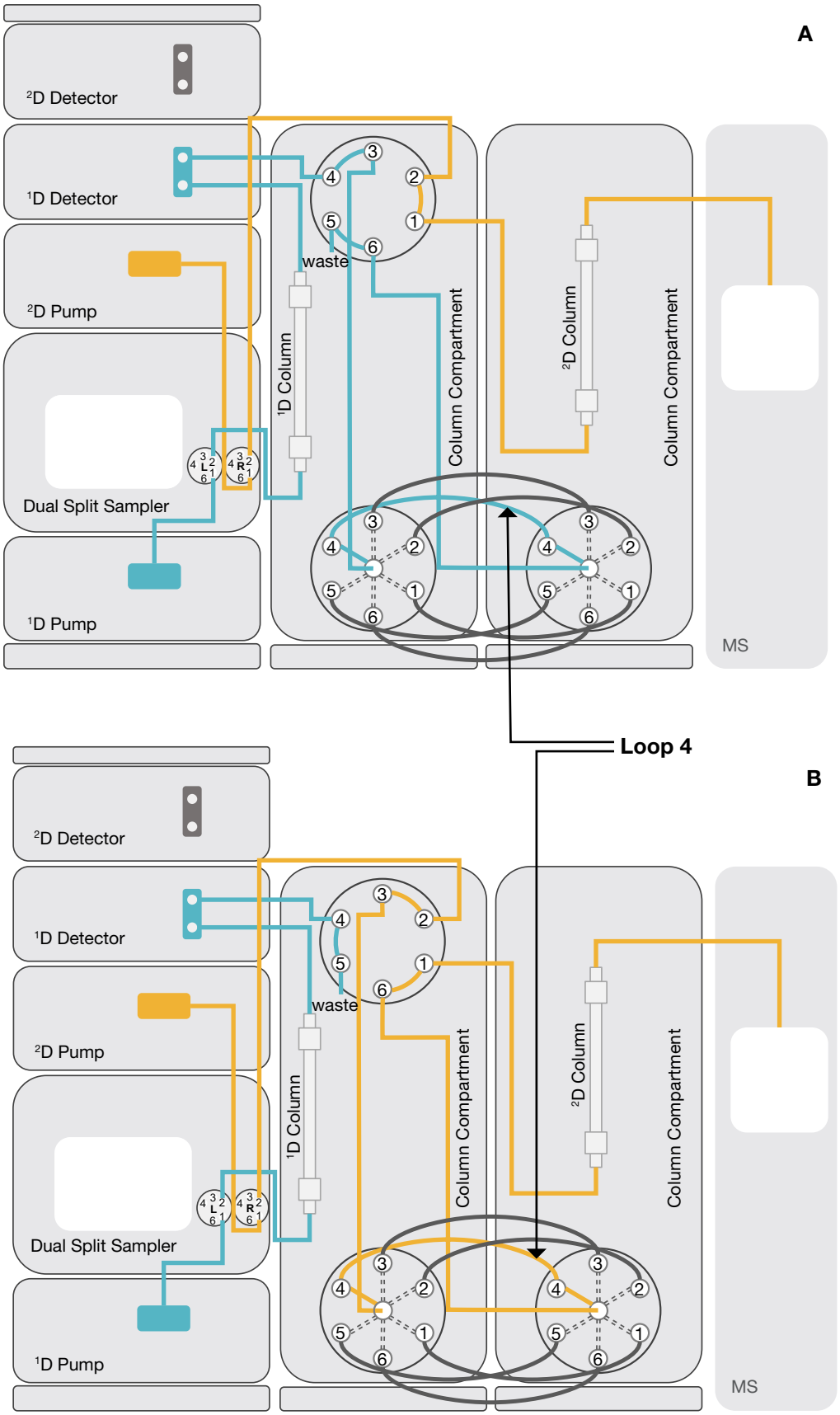

Figure 1. Fluidic configuration of the multiple loop heart-cut 2D-LC system with optional UV as ²D detector, blue = ¹D flow, yellow = ²D flow; (A) valve configuration during the ¹D exemplary loading loop 4 (2-p-6-p valve in position 1_2; both 6-p-7-p valves in position 4); (B) valve configuration during the ²D analysis of loop 4 (2-p-6-p valve in position 6_1; both 6-p-7-p valves in position 4)

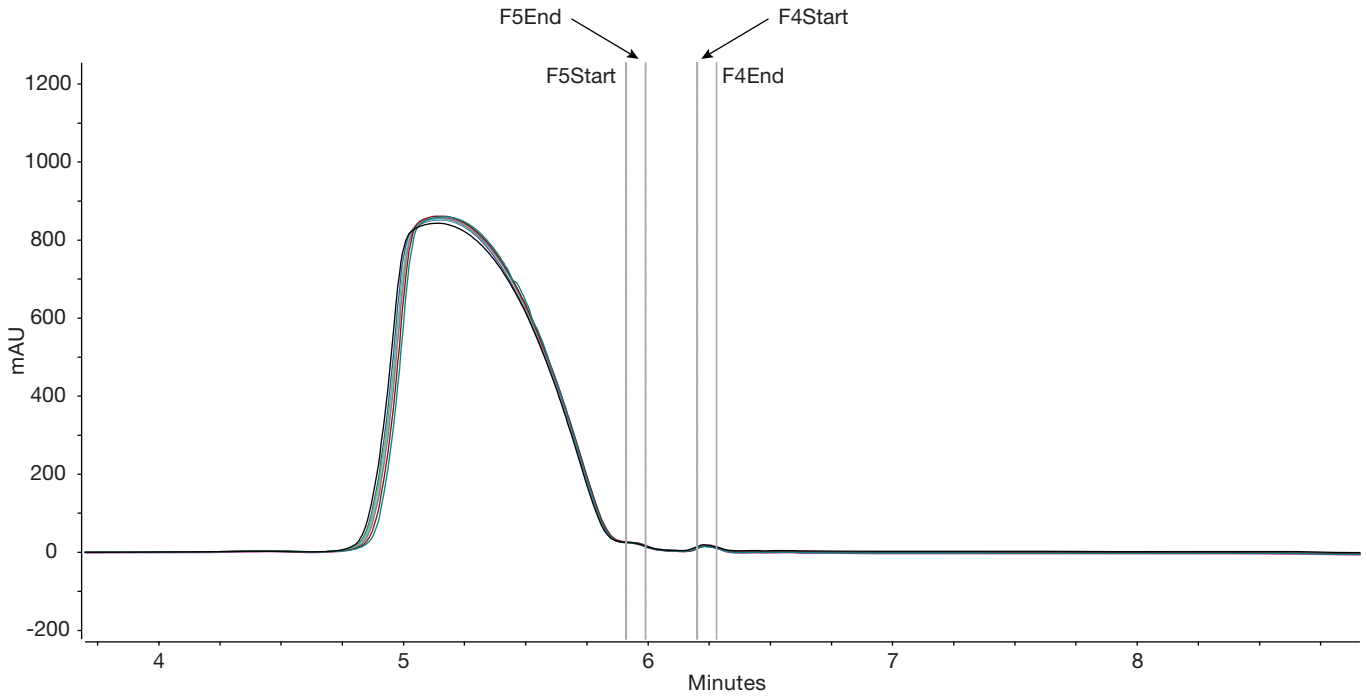

Figure 2. Overlay of eight UV chromatograms of the [1]D separation of the reduced therapeutic protein sample with indication of the two heart-cut fractions

Name	Type	*LoopD2	*F1Start [min]	*F1End [min]	*F2Start [min]	*F2End [min]	*F3Start [min]	*F3End [min]	*F4Start [min]	*F4End [min]	*F5Start [min]	*F5End [min]	Level	Position	Volume	Instrument Method
Sample 1stD	Unknown		1,00	1,10	2,00	2,10	3,00	3,10	4,00	4,10	5,00	5,10		B:B1	0,50	AP_1stD_smartcoded
Sample 2ndD_loop1	Blank	1												B:B1	0,50	AP_2ndD_smartcoded
Sample 2ndD_loop2	Blank	2												B:B1	0,50	AP_2ndD_smartcoded
Sample 2ndD_loop3	Blank	3												B:B1	0,50	AP_2ndD_smartcoded
Sample 2ndD_loop4	Blank	4												B:B1	0,50	AP_2ndD_smartcoded
Sample 2ndD_loop5	Blank	5												B:B1	0,50	AP_2ndD_smartcoded

Figure 3. Screenshot of a generic example 2D-LC sequence in Chromeleon CDS for heart-cutting and analysis of five fractions; columns "LoopD2", "FxStart", "FxEnd" were added as Custom Columns to the sequence and enable flexible control of the 2D-LC methods

1D-LC-MS and dual 1D-LC-UV on the same instrument

As mentioned earlier, even though 2D-LC instrumentation is set up in an increasing number of laboratories, 2D-LC applications often are not routinely run but applied in particular challenging analyses. The same is true for the instrument in the described biopharmaceutical laboratory. In fact, here the instrument is used frequently for 1D-LC-MS applications, which is easily done without any replumbing, as the flow path of the [2]D is equipped with a separate injection unit of the Dual Split autosampler. For example, the bottom-up peptide mapping experiment of the same therapeutic protein and its impurities, mentioned earlier, was implemented on the same instrument in 1D-LC-MS mode. Figure 4 shows the total ion chromatogram (TIC) of an 80 min RP separation of the peptide mixture obtained from the therapeutic protein sample after tryptic digestion, followed by MS detection with the instrument method comprising Full MS scans and data dependent MS^2 scans. The results from this experiment provided first insights into the identities of all impurities, which were successfully confirmed and assigned by the 2D-LC-MS approach shown above.

The valve configuration for 1D-LC-MS corresponds to Figure 1A. This feature is also useful during method development or troubleshooting of 2D-LC-MS methods, as standards can be injected directly into the [2]D column without the need of heart-cutting from the [1]D. Injection in either flow path is possible in the 2D-LC Chromeleon Instrument Configuration as well as in the Thermo Scientific™ Vanquish™ Duo for Dual LC configuration suggested in the next paragraph.

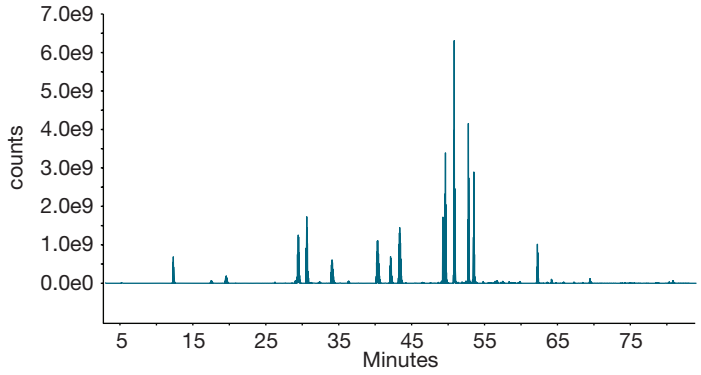

Figure 4. 1D-LC-MS total ion chromatogram of a tryptic digest of the therapeutic protein sample

Proper maintenance and regular instrument checks of laboratory equipment is one pillar of good laboratory practice to ensure reliable data, particularly in regulated environments. Periodic quality control instrument checks were executed with the LC system to ensure results within specifications. The check method includes the separation of one small molecule and one peptide with UV detection. As the 2D-LC system is equipped with one injection unit in either flow path, it can also be configured as a Vanquish Duo for Dual LC system in the Chromeleon Instrument Configuration, which means that two independent 1D-LC flow paths can be controlled independently and simultaneously, just like two instruments.[5] With this dual LC approach, both 1D-LC-UV paths can perform the instrument checks in parallel, as illustrated in Figure 5. For maximum convenience, it is recommended to save the different Chromeleon Instrument Configuration files in an easily accessible place to import and switch whenever needed.

Conclusion

- Confirmation of impurities in a therapeutic protein sample was enabled by multi heart-cut 2D-LC-MS in a top-down analysis by combining RP-LC in the ^{1}D and SEC as a TFA removing step in the ^{2}D.
- Due to the Simple Switch feature, the exact same instrument can be used for 1D-LC-MS or dual 1D-LC-UV applications, similar to the Vanquish Duo for Dual LC systems, without replumbing when 2D-LC operation is not required.
- Chromeleon CDS is the most convenient solution to control Vanquish Online 2D-LC systems.

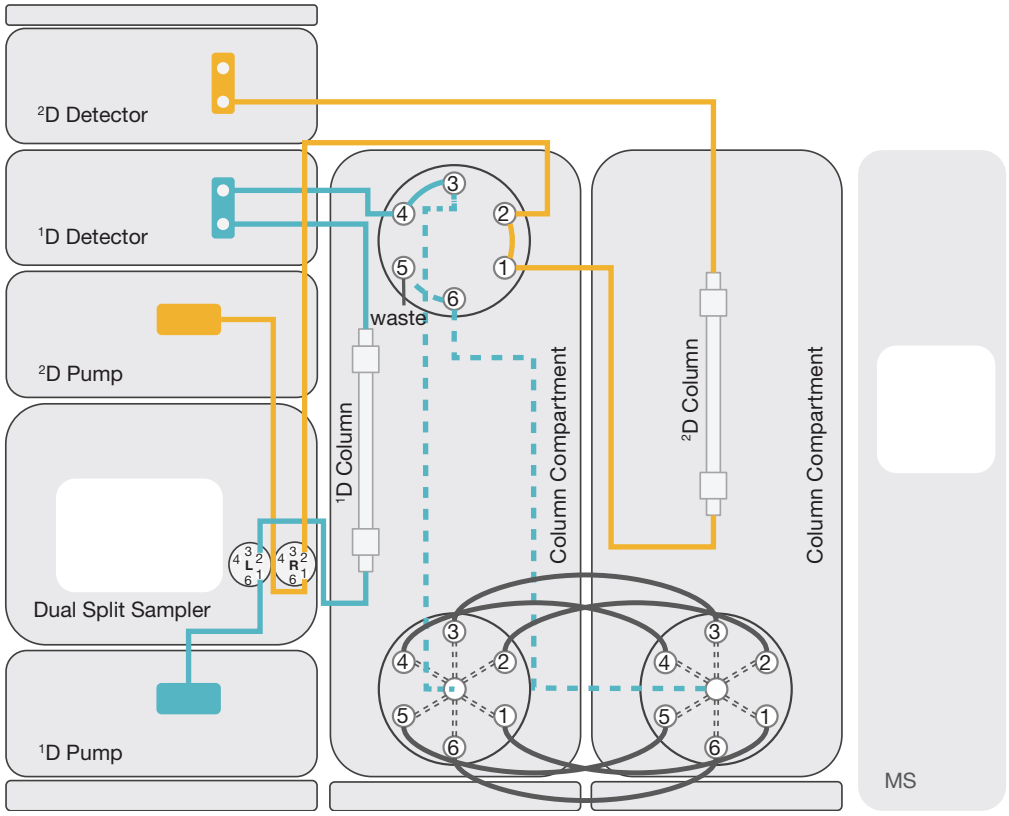

Figure 5. Fluidic configuration of the Vanquish Simple Switch 2D-LC system enabling the simultaneous operation of two 1D-LC-UV methods

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