Online 2D-LC Analysis of Complex N-Glycans in Biopharmaceuticals Using the Agilent 1290 Infinity 2D-LC Solution

Comprehensive and Multiple Heart-Cutting 2D-LC Analysis for Highest Resolution

Suitable for Agilent
1290 Infinity III LC

Application Note

Biotherapeutics & Biologics

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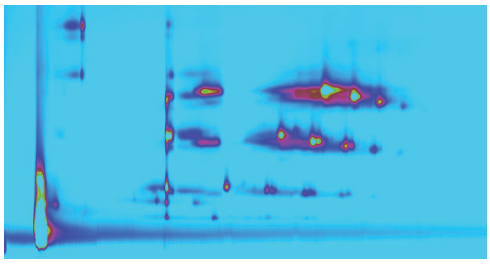
Abstract

This Application Note presents different online 2D-LC concepts for the high-resolution analysis of complex N-linked glycans in erythropoietin (EPO) using the Agilent 1290 Infinity 2D-LC Solution with fluorescence detection. EPO has a complex glycosylation pattern with differently branched and charged glycans. A combination of hydrophilic interaction chromatography (HILIC) with weak anion exchange chromatography (WAX) enables highly orthogonal separation. Comprehensive 2D-LC analysis with HILIC in the first and WAX in the second dimension facilitates high-resolution 2D chromatography together with simultaneous charge profiling. In addition, multiple heart-cutting 2D-LC analysis, combining WAX and HILIC separation, provides a flexible alternative whereby the user can select multiple peaks to be analyzed in the second dimension and, moreover, run longer gradients.



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Introduction

Erythropoietin (EPO) is a 30,400 dalton (Da) glycoprotein hormone that regulates the production of red blood cells (erythropoiesis). The molecule consists of a 165 amino acid single polypeptide chain and a complex carbohydrate addition that amounts to 40 % of the total molecular weight. Due to the flexible molecular structure of the glycans, they cover almost all of the surface of EPO[1]. The glycosylation of EPO is highly variable because it contains multiple glycosylation sites, each of which can have a wide variety of glycan structures. This results in a huge complexity of glycan structures that is referred to as microheterogeneity. The glycosylation portion of EPO consists of three N-linked glycosylation sites at Asn 24, 38, and 83, and one O-linked glycosylation site at Ser 126[1] (Figure 1). In this scheme, four differently branched and charged glycans are displayed as examples that typically occur in EPO. Each of the three N-linked glycans is likely to contain up to four sialic acids (N-acetylneuramic acid, NeuAc). The amount of NeuAcs in EPO has a huge influence on the molecule's net charge, which is used to classify EPO isoforms having a defined charge due to its sialic acid content (for example, epoetin alpha, beta, and so on).

Detailed characterization of the glycan profile of biopharmaceuticals is a regulatory requirement, as differences in glycosylation can affect both the pharmacodynamics and pharmacokinetic behavior in the human body. Therefore, it is necessary to develop advanced analytical technologies for efficient and detailed glycosylation analysis.

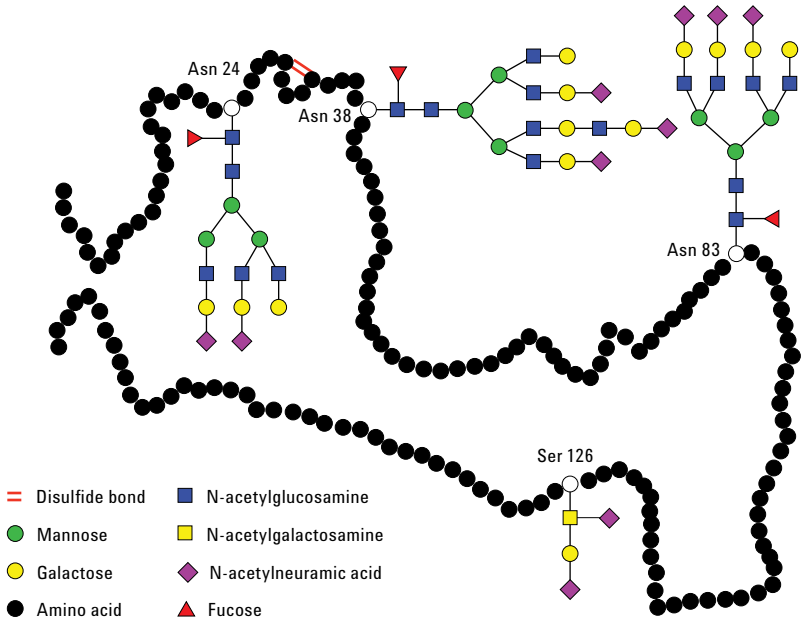

Figure 1. EPO structure with four examples of N-glycans typically occurring in EPO.

The complex mixture of highly branched glycans in EPO must first be enzymatically released from the protein before they can be studied in detail. Even after the glycans have been isolated from the protein backbone, their analysis still poses a huge analytical challenge. The method of choice is typically hydrophilic interaction chromatography (HILIC) after labelling with 2-aminobenzamide (2AB) for sensitive fluorescence detection. Whereas HILIC efficiently separates glycans according to hydrodynamic radius, it is insufficient to fully resolve the complex mixture of branched glycan structures that are present in samples such as EPO or fetuin[2]. Fortunately, weak/strong anion exchange chromatography (WAX/SAX) provides a highly orthogonal separation that depends on the number and arrangement of acidic monosaccharaides in the glycan. In the case of EPO and fetuin, these are typically sialic acids known as N-acetylneuraminic acid or NeuAc.

A combination of WAX/SAX and HILIC has a huge potential to enhance peak capacity in two-dimensional liquid chromatography (2D-LC) due to the highly orthogonal nature of these two separation techniques. Bones *et al.* showed an offline 2D analysis with a combination of WAX separation in the first dimension with fraction collection of 10 peaks, followed by HILIC analysis in the second dimension. The complete offline 2D analysis of 10 EPO N-glycan peaks resulted in a total cycle time of over 4 hours with additional hands-on time for moving the samples from the fraction collector into a sample injector for re-injection. This type of 2D workflow typically also requires additional time to dry down the first dimension fractions in a vacuum centrifuge prior to resuspending them in a suitable matrix and volume for the second dimension analysis.

The Agilent 1290 Infinity 2D-LC solution enables online 2D-LC workflows for either comprehensive or (multiple) heart-cutting analysis. Comprehensive 2D-LC analysis, using two sample loops within a 2-position/4-port-duo valve, captures all peaks from the first dimension. If higher resolution in the second dimension is desired, the Agilent 1290 Infinity multiple heart-cutting 2D-LC solution enables more flexibility, for example, longer cycle times or columns. This solution is comprised of two external valve drives with 6-position/-14-port valves, each with six pre-installed 40-µL loops, resulting in twelve loops. Figure 2 shows an Agilent multiple heart-cutting valve with pre-installed loops.

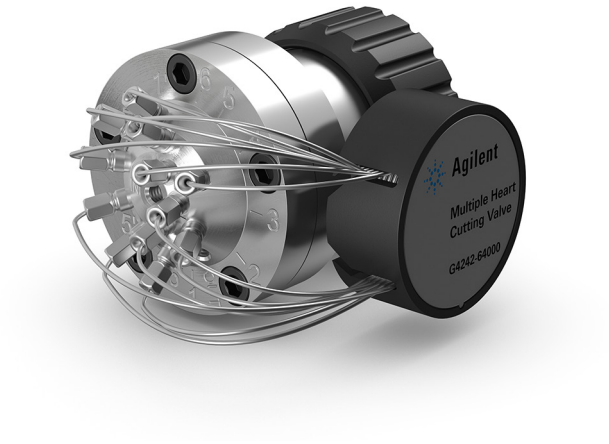

Figure 2. Agilent Multiple Heart Cutting Valve with six pre-installed 40-µL loops.

This Application Note shows online 2D-LC concepts for N-glycan analysis of therapeutic EPO using a combination of HILIC/WAX for comprehensive analysis and WAX/HILIC for detailed multiple heart-cutting analysis.

Experimental

The Agilent 1290 Infinity 2D-LC solution was comprised of the following modules:

- Agilent 1260 Infinity Bio-inert Quaternary Pump (G5611A) for the first dimension
- Agilent 1290 Infinity Binary Pump (G4220A) for the second dimension
- Agilent 1290 Infinity Autosampler (G4226A) with an Agilent 1290 Infinity Thermostat (G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1290 Infinity Valve Drive (G1170A) with 2-position/4-port duo valve (G4236A) equipped with either two 40-µL loops, or
- Agilent Multiple Heart Cutting Valve Upgrade Kit (G4242A)
- Agilent 1260 Infinity Fluorescence Detector (G1321B), with standard 8-mm flow cell

MS system

Agilent 6530 Accurate-Mass QTOF LC/MS system

Columns

- Agilent AdvanceBio Glycan Mapping column, 2.1 × 150 mm, 1.8 µm (p/n 859700-913)
- Agilent AdvanceBio Glycan Mapping column, 4.6 × 50 mm, 2.7 µm (custom)
- Agilent Bio WAX column, 2.1 × 250 mm, 5 µm (p/n 5190-2491)
- Agilent Bio WAX column, 2.1 × 50 mm, 5 µm (p/n 5190-2492)

Software

- Agilent OpenLAB CDS ChemStation Edition for LC & LC/MS Systems Rev. C.01.06 [61]
- Agilent MassHunter Workstation software, Version B.05.01, Build 4.0.479.0
- Glycan structures were created with GlycoWorkbench, Version 2.1, stable (146)

Solvents and samples

All solvents used were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22-µm membrane point-of-use cartridge (Millipak). Ammonium formate, fetuin, and ovalbumin, PNGase F from Elizabethkingia miricola, GlycoProfil 2-AB Labeling Kit and GlycoProfil Glycan Cleanup Cartridges were purchased from Sigma-Aldrich, St.Louis, USA. B-EPO was used as example of a therapeutic EPO.

Chromatographic conditions

Comprehensive 2D-LC analysis HILIC/WAX	
Valve and loop configuration	2-position/4-port duo valve, 2 loops (concurrent), loop size 40 µL
1D mobile phase A	Acetonitrile
1D mobile phase B	10 mM ammonium formate in water, pH 4.5
2D mobile phase A	40 % acetonitrile
2D mobile phase B	40 % acetonitrile, 250 mM ammonium formate, pH 4.5
1D flow rate	0.05 mL/min
1D gradient for EPO	28 % B at 0 minutes 47 % B at 110 minutes 100 % B at 110 minutes
1D gradient for Fetuin	30 % B at 0 minutes 44 % B at 110 minutes 100 % B at 110 minutes
1D stop time	120 minutes
1D post time	45 minutes
2D parameter mode	Comprehensive
2D gradient stop time	0.36 minutes
Modulation time	0.50 minutes
Flow	1.50 mL/min
Idle flow	0.50 mL/min
2D gradient	5 % B at 0.00 minutes 95 % B at 0.35 minutes 5 % B at 0.36 minutes
Injection volume	20 µL, 3 µL
Thermostat autosampler	5 °C
Column temperature 1D column Column temperature 2D column	40 °C 50 °C
FLD	Excitation 260 nm, emission 430 nm
Peak width	> 0.0031 minutes (0.063 s response time), 37.04 Hz
MS parameters	
Gas temperature	250 °C
Sheath gas temperature	250 °C
Gas flow	8 L/min
Sheath gas flow	8 L/min
Nebulizer	25 psi
V_{cap}	3,500 V
Nozzle	1,000 V
Skimmer	45 V
Oct 1 RF Vpp	550
Mode	MS

Multiple heart-cutting 2D-LC analysis WAX/HILIC	
1D mobile phase A	40 % acetonitrile
1D mobile phase B	40 % acetonitrile, 250 mM ammonium formate, pH 4.5
2D mobile phase A	Acetonitrile
2D mobile phase B	50 mM ammonium formate in water, pH 4.5
Valve and loop configuration	2-position/4-port duo valve, 2 × 6 loops (concurrent), loop size 40 µL
1D flow rate	0.25 mL/min
1D gradient for EPO	5 % B at 0 minutes 60 % B at 20 minutes 5 % B at 21 minutes
1D stop time	72 minutes
2D parameter mode	Multiple heart-cutting
2D gradient stop time	3.5 minutes
2D cycle time	4.9 minutes
Flow	2 mL/min
Idle flow	0.50 mL/min
2D gradient	65 % B at 0.00 minutes 57 % B at 3.5 minutes
2D time segments (heart cutting time points) time-based, loop fill time 0.1 minutes	6.85 minutes, 7.45 minutes, 7.85 minutes, 11.15 minutes, 11.45 minutes, 11.75 minutes, 12.30 minutes, 14.60 minutes, 14.85 minutes, 15.25 minutes, 15.70 minutes
Injection volume	20 µL
Thermostat autosampler	5 °C
Column temperature 1D column	25 °C
Column temperature 2D column	40 °C
FLD	Excitation 260 nm, emission 430 nm
Peak width	0.025 minutes (0.5 seconds response time), 18.52 Hz

Results and Discussion

Comprehensive 2D-LC analysis of complex N-glycans

Before analysis, N-glycans from fetuin and EPO were enzymatically released and labelled with 2-AB for sensitive fluorescence detection. For method development, N-glycans from fetuin were used, as the glycosylation profiles of fetuin and EPO are similar regarding the sample complexity and presence of highly branched and sialylated N-glycans. The Agilent AdvanceBio Glycan Mapping column demonstrated excellent resolving power for different glycoproteins such as monoclonal antibodies and others[3]. However, the separation within a one-dimensional HILIC setup is not sufficient to resolve all N-glycans of EPO or fetuin (Figure 3).

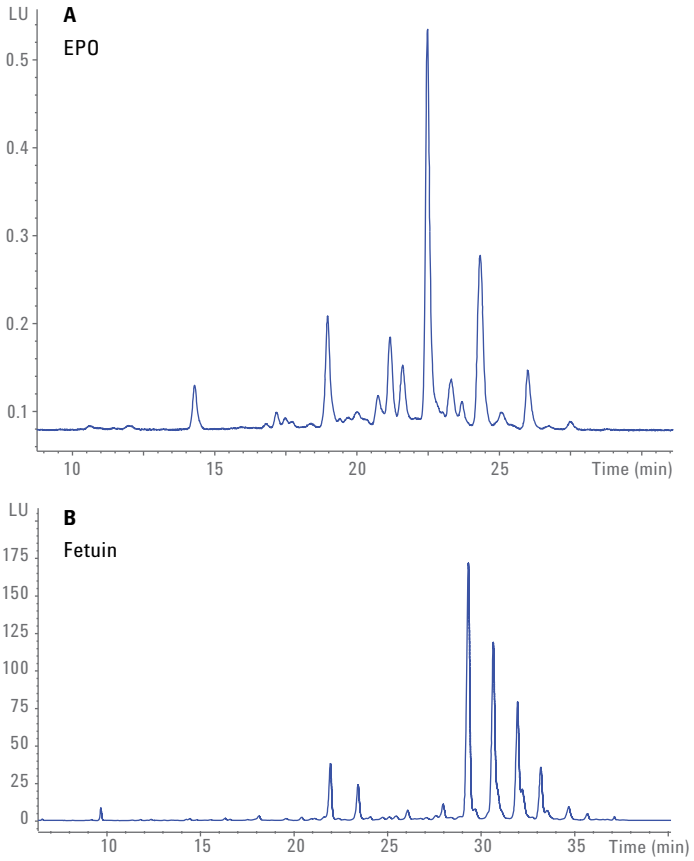

Figure 3. HILIC separation on an Agilent AdvanceBio Glycan Mapping Column, 2.1 × 150 mm, 1.8 µm.

To improve resolution and enhance peak capacity, a combination of WAX and HILIC separation was used for a highly orthogonal separation. First, a comprehensive WAX/HILIC 2D-LC setup was tested using fetuin. A 110-minute WAX gradient was used for the first dimension, followed by a 30-second second-dimension comprehensive HILIC run using a 4.6 × 50 mm HILIC column. Although 40 % ACN was used for the first-dimension solvents, the glycans were not retained on the HILIC column (data not shown). Typically, HILIC columns require a longer re-equilibration time than other types of columns. Presumably, the 30-second cycle times in the second dimension are not compatible with HILIC separations of glycans. Moreover, the high amount of water (60 %) in the first-dimension effluent, together with a relatively high loop volume of 40 µL and the very short gradient, did not allow good glycan retention on the short 50-mm HILIC column. Therefore, the order of the dimensions was reversed, and a comprehensive HILIC/WAX separation was used in an online 2D-LC setup, maintaining the highly orthogonal separation. Figure 4 shows the 2D-LC image from a HILIC/WAX 2D run. The 2D separation provides high peak capacity and resolution, and many of the coeluting peaks from the HILIC dimension are well separated by WAX.

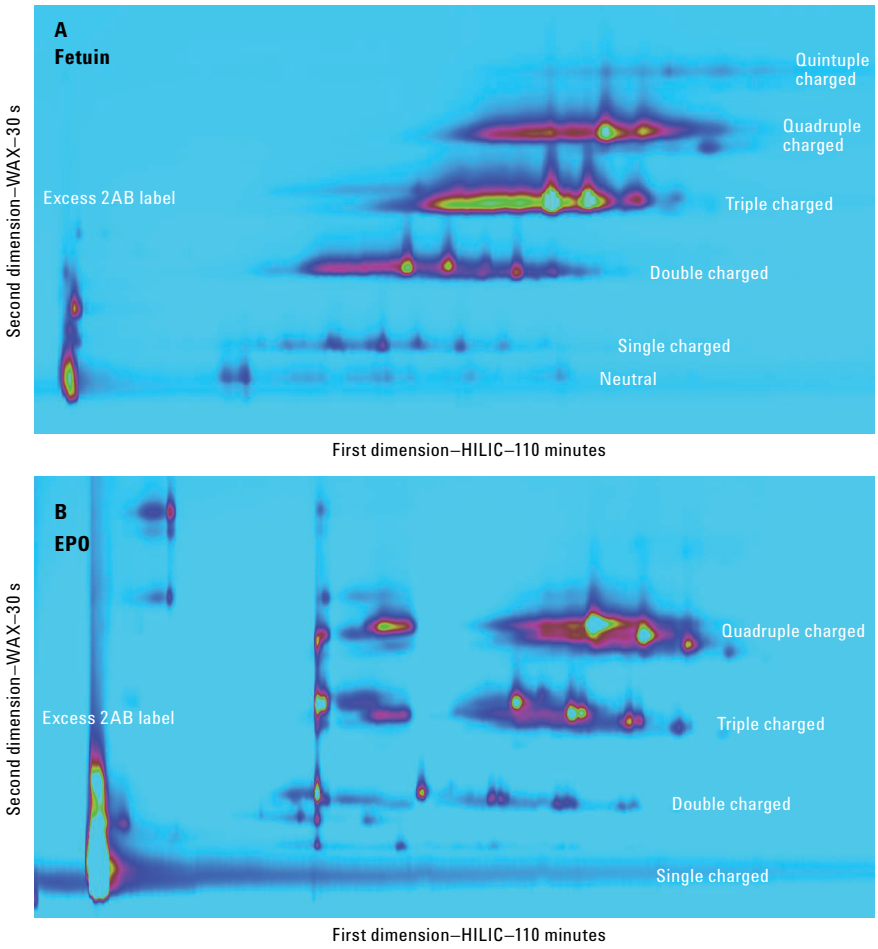

Figure 4. Comprehensive HILIC/WAX 2D-LC separation of fetuin and EPO, showing highly orthogonal separation. The ion exchange chromatography in the second dimension reveals the charge pattern of the glycans.

With parallel MS/Q-TOF analysis, the peaks were assigned to the corresponding charge (which for most peaks is equal to the number of sialic acids contained in the glycan). The detected parent ion masses were entered into the GlycoMod tool from Expasy to find the related glycans structures (http://web.expasy.org/glycomod/, accessed November 1, 2014). As expected, the second-dimension separation groups glycans according to their charge. The neutral glycans, which elute immediately with the injection peak, are shortly followed by the singly charged glycans. More clearly separated, the double, triple, quadruple, and a few (fetuin) quintuple-charged glycans elute with an increasing salt gradient in the second dimension. Therefore, in addition to increasing the peak capacity, the WAX separation assists peak assignment, and furthermore provides the glycan charge profile that is required in the analysis of EPO glycosylation. As mentioned above, EPO isoforms are classified according to their net charge (epoetin alpha, beta, and so on). This setup enables simultaneous charge profiling in combination with a well resolved glycan peak pattern.

Multiple heart-cutting 2D-LC analysis of complex N-glycans

If WAX in the first dimension is implicitly favored, the Agilent multiple heart-cutting solution can be used, maintaining the HILIC column in the second dimension. Due to the multiple heart-cutting setup using twelve 40-µL loops in two 6-position/14-port valves (Figure 5), it is possible to *par*k peaks from the first dimension, enabling longer second-dimension gradients.

The chromatogram of the first dimension separation can be loaded into the 2D-LC acquisition setup to facilitate method development (Figure 6). The peaks that were chosen to be re-analyzed, can be selected either by peak triggering using a first-dimension detector, or using time segments with certain loop fill times. This enables the reduction of solvent from the first dimension. The HILIC gradient in the second dimension starts with a total amount of 35 % water. If too much water is injected into the second-dimension column, glycan retention can be reduced. The amount of water from the first dimension eluent can be reduced, if the 40-µL loops are only partly filled with the peaks from the first dimension. Here, the loops are filled 62.5 % with solvent from the first dimension, lowering the total amount of water that is injected into the second-dimension column.

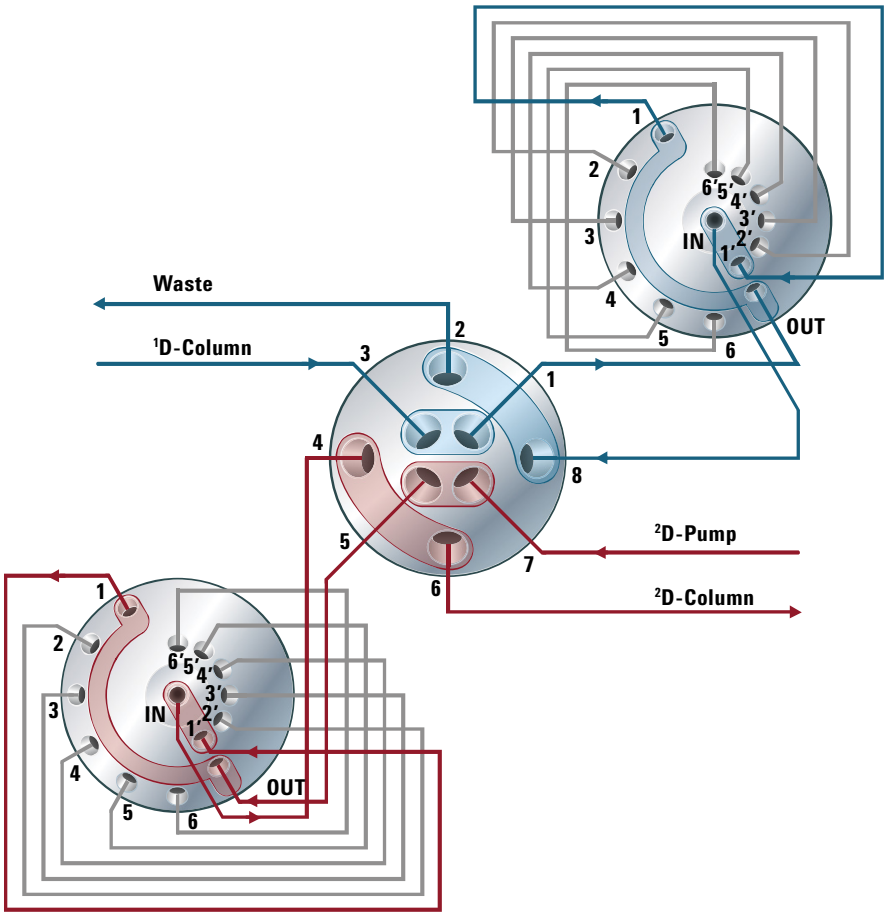

Figure 5. Plumbing diagram of the 2-position/4-port duo valve in combination with two 6-position/14-port valves with 12 pre-installed 40 µL loops.

Figure 6 shows the 2D pump setup with the loaded first-dimension chromatogram with 11 peaks to be re-analyzed (yellow marks), also represented by 11 2D time segments (red box). Although only 10 loops are available for storing the peaks, while two loops are always in the flow path, this setup enables the analysis of more than 10 peaks. After the first peak is collected in the first loop of the first 6-position/14-port valve, it is immediately injected onto the second-dimension column by switching the central 2D-LC valve. After the 2D-LC valve has switched, the loops of the second 6-position/14-port valve can be filled with up to five peaks. As soon as the first 2D gradient has finished, the 2D-LC valve switches back so that the loops of the first 6-position/14-port valve can be filled. This, however, requires that the 2D analysis of the first peak is finished. Therefore, for method development, the adjustment of cycle time in the second dimension is critical for flexible peak selection.

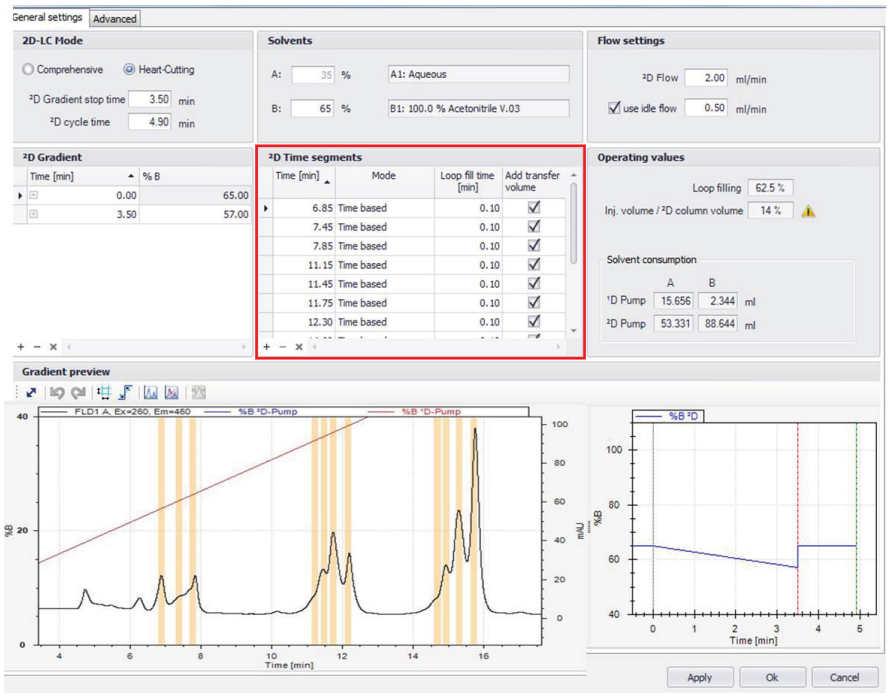

Figure 6. Setup of the 2D pump, showing the loaded WAX chromatogram from the first dimension with 11 peaks chosen for re-analysis in the second dimension using HILIC. Eleven 2D time segments represent the chosen peak areas (red box).

In contrast to the comprehensive 2D-LC solution, the multiple heart-cutting solution allows analysis by HILIC in the second dimension. This is because the multiple heart-cutting approach allows the use of longer gradient and re-equilibration times in the second dimension. In this experiment, a gradient time of 3.5 minutes was used with a re-equilibration time of 1.4 minutes. The glycans were retained on the short HILIC column and a good 2D resolution was achieved (Figure 7). Six examples are shown to demonstrate the resolving power of the HILIC separation within the multiple heart-cutting setup (peaks 1, 4, 5, 8, 9, and 10). Areas that are only visible as shoulders in the first dimension, for example, peak 8, revealed at least eight peaks in the second dimension. Under most of the peaks, which are only showing one major peak in the first dimension, several underlying peaks were detected and resolved.

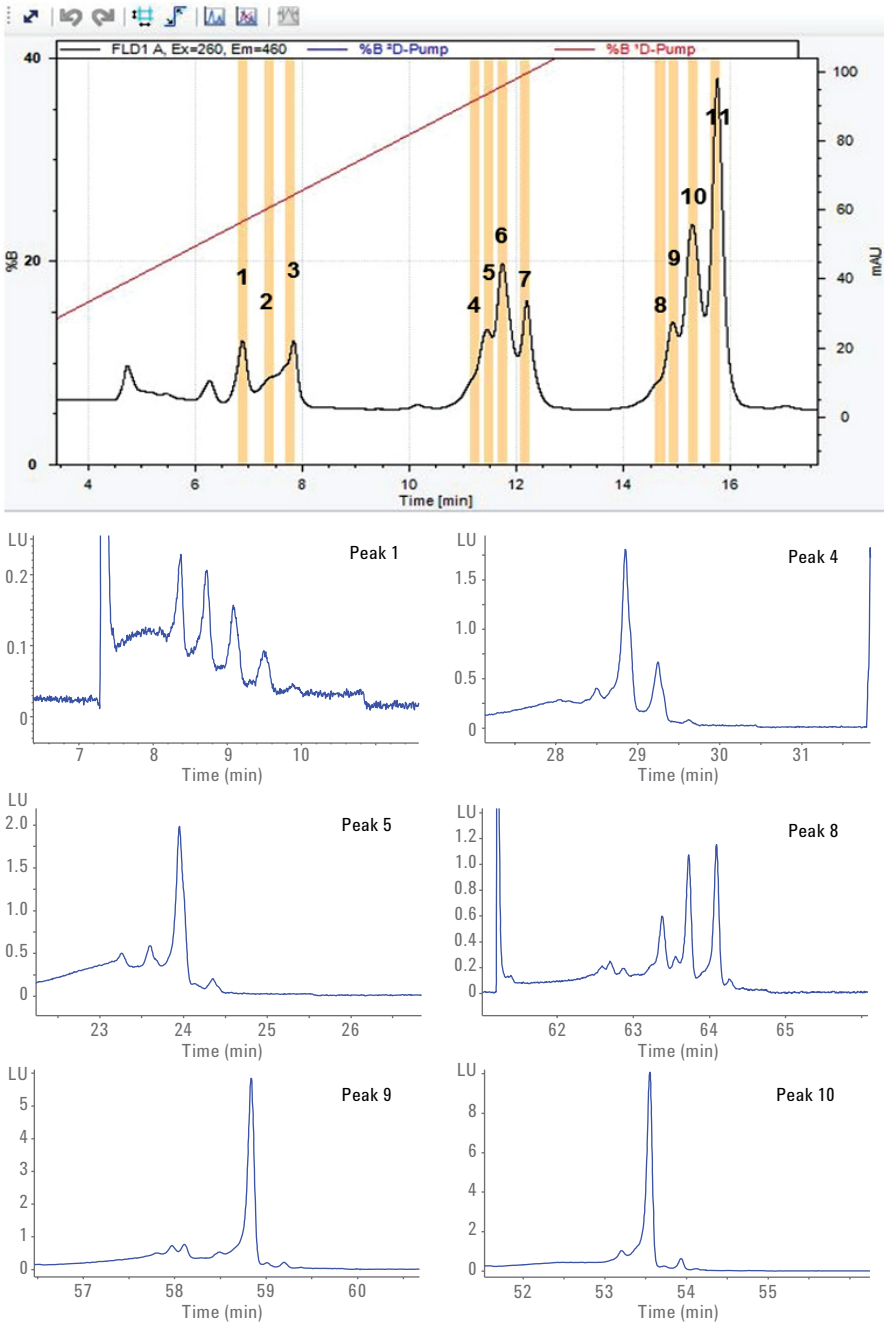

Figure 7. Six examples for the high resolving power of the multiple heart-cutting setup, resolving up to eight peaks under the area that is marked with number 8.

Conclusion

This Application Note shows different concepts for online 2D-LC analysis of complex N-glycans found in therapeutic EPO using the Agilent 1290 Infinity 2D-LC Solution with fluorescence detection. Differently branched and charged glycans are attached to EPO resulting in a highly complex and heterogeneous glycan profile, in which especially the net charge of EPO is used for the classification of therapeutic EPO. The complexity of the EPO glycans requires modern analysis techniques that deliver high resolution.

Although current HILIC columns have a high resolving power for glycans, the mixture of glycans on therapeutic EPO is so complicated that full resolution cannot be achieved by a one-dimensional HILIC separation. Online comprehensive 2D-LC with a combination of HILIC in the first dimension and a highly orthogonal WAX separation in the second dimension provides high resolution and peak capacity. Complete automation of the 2D analysis enables a run time of only 110 minutes when compared to a much longer run time for offline analysis. Furthermore, the resulting data are easy to interpret because the glycans are grouped according to their charge in the second dimension, satisfying the requirement of charge profiling for EPO glycans.

The multiple heart-cutting solution offers greater flexibility and facilitates the more technically demanding combination of WAX in the first, and HILIC in the second dimension. With this 2D separation method, the user is able to select several peaks from the WAX chromatogram for additional separation in the second dimension. In addition, because the peaks are parked in the 40-µL loops of the two 6-position/14-port valves, their second-dimension separation is no longer limited to super short gradients. Instead, the cycle time for the second dimension can be adjusted as needed, depending on the distribution of the peaks within the first-dimension chromatogram. In comparison to offline 2D-LC using WAX/HILIC analysis with fraction collection (Bones *et al.*), a time savings of about 70 % was possible, reducing the total analysis time from over 4 hours down to about 70 minutes.

The 1290 Infinity 2D-LC Solution together with the Agilent 1260 Infinity Fluorescence Detector and the combination of HILIC and WAX separation media enable excellent resolving power for highly complex glycans from therapeutic glycoproteins.

References

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