



# 2D-LC as an Automated Desalting Tool for MSD Analysis

## Direct Mass Selective Detection of a Pharmaceutical Peptide from an MS-Incompatible USP Method

### Application Note

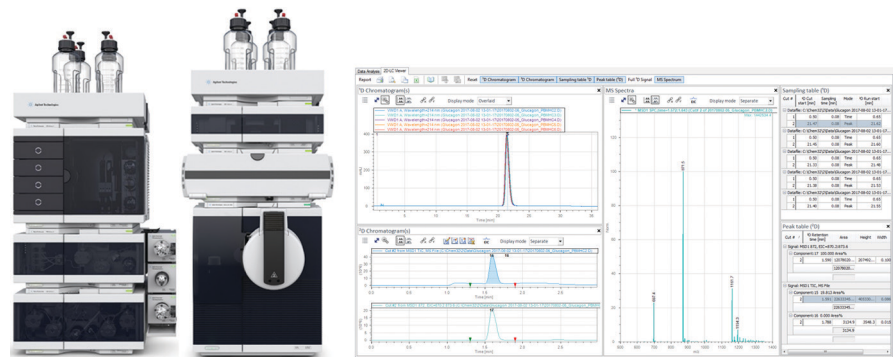
Biologics and Biosimilars

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#### Abstract

The characterization and impurity analysis of therapeutic peptides and proteins requires the use of chromatographic separation combined with mass selective detection. Chromatographic separation methods for peptides and proteins, however, often use MS-incompatible mobile phases. This Application Note demonstrates the use of multiple heart-cutting (MHC) two-dimensional liquid chromatography (2D-LC) as an automated desalting tool for mass selective detection. The peptide glucagon was analyzed in accordance with USP 39 in the first dimension, using an MS-incompatible mobile phase. Automated desalting and mass selective detection were then performed in the second dimension.



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## Introduction

There is increasing interest in therapeutic peptides and proteins in pharmaceutical research and development. The complicated structure and impurity profile of these larger molecules leads to challenges associated with their characterization, and the analysis of impurities. These challenges require the use of chromatographic separation combined with mass selective detection (MSD) for the analysis of these molecules<sup>1</sup>. Chromatographic separation methods for peptides and proteins often use MS-incompatible mobile phases, for example, those containing concentrated salts or nonvolatile buffers<sup>1,2</sup>. To enable MS detection for these chromatographic methods, method translation to MS-compatible mobile phases or collection of fractions with offline MS analysis after desalting is generally required<sup>1</sup>.

In two-dimensional liquid chromatography (2D-LC), the second dimension can be used as an effective desalting tool to allow online coupling of chromatographic methods using MS-incompatible mobile phases to MS detection<sup>1</sup>. The Agilent InfinityLab 2D-LC Solution with 2D-LC Software A.01.04 offers a fully integrated solution from method setup to data analysis for 2D-LC with MSD using Agilent single quadrupole mass spectrometers. A diverter valve can be used to automatically divert salt or buffers originating from the first-dimension (<sup>1</sup>D) mobile phase to waste at the beginning of every second-dimension (<sup>2</sup>D) run. Agilent AdvanceBio Desalting-RP cartridges can be used to provide fast and effective desalting in the second dimension, as shown in a previous Application Note<sup>3</sup>. In addition to desalting, further separation can be achieved in the second dimension when full-length columns and conditions providing selectivity differences compared to the first dimension separation are used<sup>2</sup>.

Glucagon is a peptide hormone containing 29 amino acids that is used for the emergency treatment of hypoglycemia<sup>4</sup>. As per USP 39, the assay and analysis of glucagon for organic impurities is performed by LC with UV detection using potassium phosphate buffer in the mobile phase<sup>5</sup>. This is not compatible with MS detection. This Application Note demonstrates the use of multiple heart-cutting (MHC) 2D-LC for the analysis of glucagon according to USP 39 in the first dimension with automated desalting and MSD in the second dimension.

## Experimental

### Equipment

The Agilent InfinityLab 2D-LC Solution comprised the following modules:

- Two Agilent 1290 Infinity II High Speed Pumps (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167B) with cooler (option #100)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)
- Agilent 1290 Infinity II Variable Wavelength Detector (G7114B) with standard flow cell (p/n G1314-60186)
- Agilent 1290 Infinity Valve Drive (G1170A) with 2-position/4-port duo valve head (2D-LC valve 1,300 bar: p/n 5067-4244)
- Two Agilent 1290 Infinity Valve Drives (G1170A) with multiple heart-cutting valves (p/n G4242-64000) equipped with 40  $\mu$ L loops
- Agilent 1290 Infinity Valve Drive (G1170A) with 2-position/6-port valve 800 bar (p/n 5067-4282)

Mass selective detection was performed using an Agilent 6150 Single Quadrupole LC/MS (G6150BA) equipped with an Agilent Jet Stream ESI source (p/n 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 C18, 3.0  $\times$  150 mm, 3.5  $\mu$ m (p/n 959963-302)
- Agilent AdvanceBio Desalting-RP, 2.1  $\times$  12.5 mm (p/n PL1612-1102) with cartridge holder (p/n 820999 901)

### Chemicals

All solvents were LC grade. Acetonitrile was purchased from Merck (Darmstadt, Germany). Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22- $\mu$ m membrane point-of-use cartridge (Millipak, EMD Millipore, Billerica, MA, USA). Potassium phosphate monobasic, formic acid, and glucagon (synthetic powder, cell culture tested) were purchased from Sigma-Aldrich (Steinheim, Germany). Hydrochloric acid and phosphoric acid were obtained from Merck (Darmstadt, Germany) and J.T. Baker (Deventer, Netherlands), respectively.

### Sample

A standard solution of glucagon at a concentration of approximately 0.5 mg/mL was obtained by reconstitution of glucagon in 0.01 N HCl. To obtain the glucagon system suitability solution per USP 39 instructions<sup>5</sup>, a solution of approximately 0.5 mg/mL of glucagon in 0.01 N HCl was kept at 50  $^{\circ}$ C for 48 hours.

## Method

First dimension	
Column	Agilent ZORBAX Eclipse Plus C18, 3.0 × 150 mm, 3.5 μm
Solvent	A) 16.3 g KH <sub>2</sub> PO <sub>4</sub> in 800 mL water adjusted to pH 2.7/200 mL acetonitrile B) Water/acetonitrile (60/40)
Gradient	0 minutes – 33.80 %B 30 minutes – 33.80 %B 34 minutes – 88.00 %B 35 minutes – 88.00 %B 36 minutes – 33.80 %B
Stop time	36 minutes
Post time	40 minutes
Stop time and post time were set in the <sup>2</sup> D pump to allow extension of the run time until completion of <sup>2</sup> D analysis of all cuts.	
Flow rate	0.500 mL/min
Temperature	45 °C
Detection	VWD, 214 nm, signal peak width: >0.025 minutes (20 Hz) To protect the flow cell from pressure pulses originating from valve switches, a pressure release kit (G4236-60010) was installed between the VWD and the 2D-LC valve.
Injection	Injection volume: 15 μL Sample temperature: 8 °C Needle wash: 3 seconds in water/acetonitrile (70/30)

Second dimension	
The <sup>2</sup> D pump was connected to the 2D-LC valve using a 0.075 × 340 mm restriction capillary (p/n 5067-4783) to provide sufficient backpressure.	
Column	Agilent AdvanceBio Desalting-RP, 2.1 × 12.5 mm
Solvent	A) Water + 0.1 % formic acid B) Acetonitrile + 0.1 % formic acid
Gradient	0.00 minutes – 5 %B 1.00 minute – 5 %B 1.50 minutes – 80 %B 2D gradient stop time: 2.00 minutes 2D cycle time: 3.00 minutes
Flow rate	0.400 mL/min
Temperature	Not controlled
Detection	Mass selective detection (MSD) A 2-position/6-port valve was installed as diverter valve to switch between directing the flow to the MSD and to waste.

MSD		
MSD Signals	Polarity	Positive
	Mode	Scan
	Mass range	600–1,350 <i>m/z</i>
	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 <sup>2</sup> 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

Peak-based multiple heart-cutting 2D-LC	
2D-LC mode	Heart-cutting
Sampling table	0.50 minutes time-based, with 0.08 minutes sampling time 19.00 minutes peak-based, with 0.65 minutes sampling time 22.50 minutes peak-based, with 0.40 minutes sampling time
Peak detection	Peak detection mode: Threshold Threshold: 5 mAU
Diverter valve	Switch to MSD after 1.00 minute
Time-based multiple heart-cutting 2D-LC	
2D-LC mode	Heart-cutting
Sampling table	The following sampling table was set up using the <sup>1</sup> D chromatogram of the glucagon system suitability solution as reference chromatogram: 0.50 minutes time-based, with 0.08 minutes sampling time 1.55 minutes time-based, with 0.08 minutes sampling time 3.70 minutes time-based, with 0.08 minutes sampling time 7.23 minutes time-based, with 0.08 minutes sampling time 11.07 minutes time-based, with 0.08 minutes sampling time 17.70 minutes time-based, with 0.08 minutes sampling time 21.38 minutes time-based, with 0.08 minutes sampling time 23.81 minutes time-based, with 0.08 minutes sampling time 24.91 minutes time-based, with 0.08 minutes sampling time 29.45 minutes time-based, with 0.08 minutes sampling time 30.25 minutes time-based, with 0.08 minutes sampling time
Diverter valve	Switch to MSD after 1.00 minute

## Results and Discussion

The analysis of glucagon for organic impurities according to USP 39 is performed by LC with UV detection using potassium phosphate buffer in the mobile phase<sup>5</sup>. This is not compatible with MS detection. Multiple heart-cutting

2D-LC can be used to allow online coupling of this LC method to MS detection, using the second dimension as an effective desalting tool. The Agilent InfinityLab 2D-LC Solution with 2D-LC Software A.01.04 provides a fully integrated solution for 2D-LC with MSD using Agilent single quadrupole mass

spectrometers. To facilitate desalting before MS detection, a diverter valve can be used to automatically divert salt or buffers originating from the 1D mobile phase to waste at the beginning of every 2D run, as shown in Figure 1.

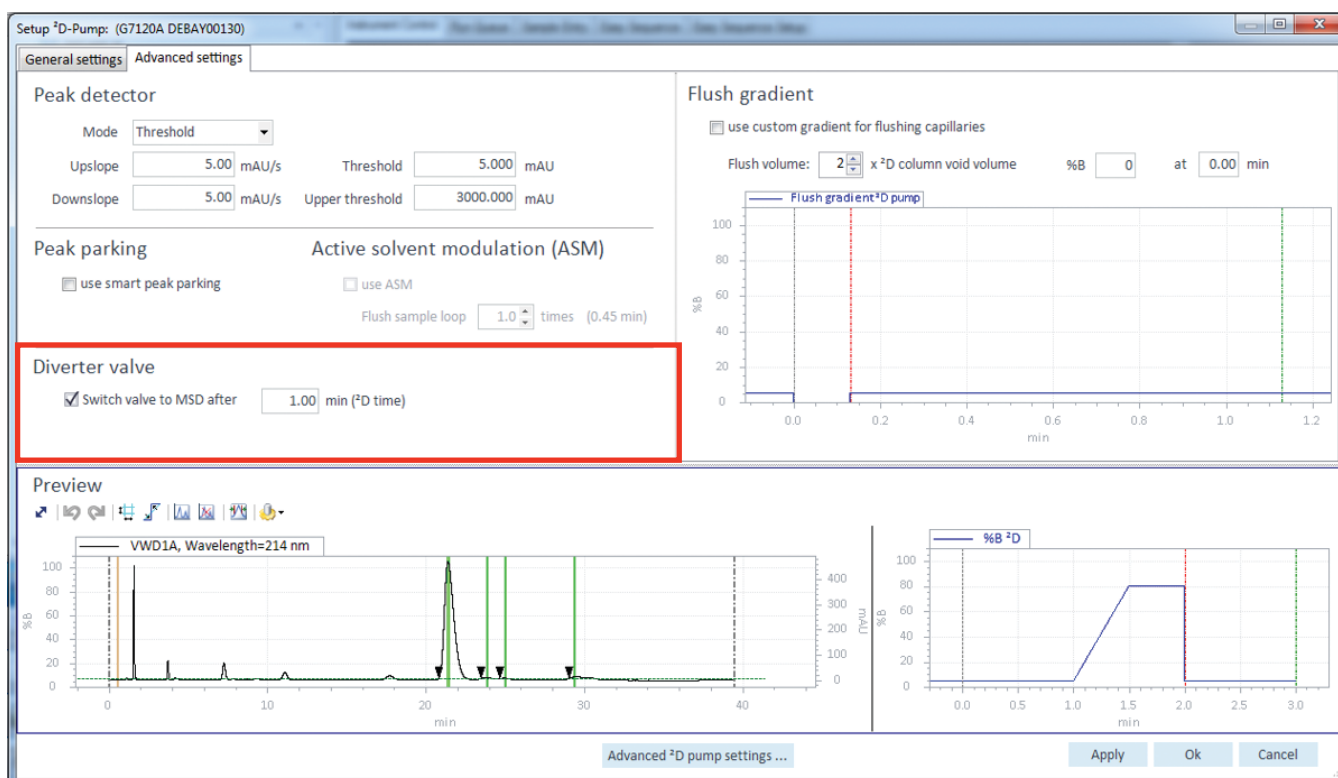


Figure 1. 2D-LC method setup for automatically diverting salt or buffers originating from the 1D mobile phase to waste at the beginning of every 2D run.

Figure 2 shows the result of the peak-based multiple heart-cutting 2D-LC analysis of the glucagon standard solution in the 2D-LC viewer. The 2D-LC viewer displays an overlay of five <sup>1</sup>D chromatograms of the glucagon standard solution and the corresponding

<sup>1</sup>D sampling table. The 2D-LC viewer also presents the <sup>2</sup>D MSD chromatogram of one <sup>2</sup>D analysis of the glucagon peak showing the total ion chromatogram (TIC) as well as an extracted ion chromatogram (EIC) and the corresponding mass spectrum.

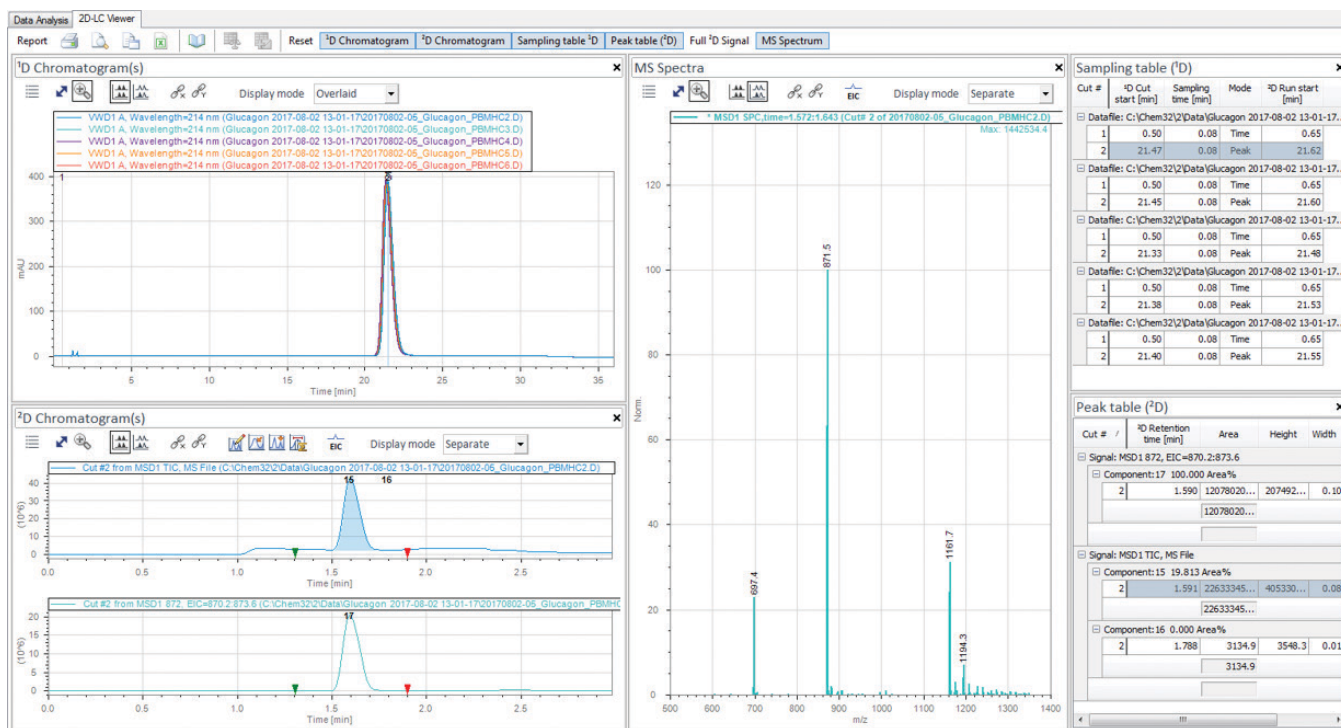


Figure 2. 2D-LC Viewer showing the results of the peak-based multiple heart-cutting 2D-LC analysis of the glucagon standard solution.

The analysis of glucagon according to USP 39 demands that certain system suitability requirements concerning the analysis of the glucagon standard and system suitability solutions are fulfilled. Figure 3 shows the 1D chromatograms of the fivefold analysis of the glucagon standard solution and the analysis of the glucagon system suitability solution. In the glucagon system suitability solution, four peaks eluting after the glucagon peak are clearly visible, which, per USP 39, originate from the deamidation of glucagon. Deamidation is the loss of ammonia from asparagine or glutamine to form aspartic or glutamic acid, respectively. This formation of a side chain carboxylic acid residue from a side chain amide<sup>4</sup> leads to a +1 Da mass increase. For example, deamidation is a common degradation pathway of proteins, which occurs during thermal stressing<sup>4</sup>. The peptide glucagon contains one asparagine and three glutamine residues.

Table 1 summarizes the system suitability requirements according to USP 39 and shows that these requirements are excellently fulfilled by the analysis performed in the first dimension of the 2D-LC method.

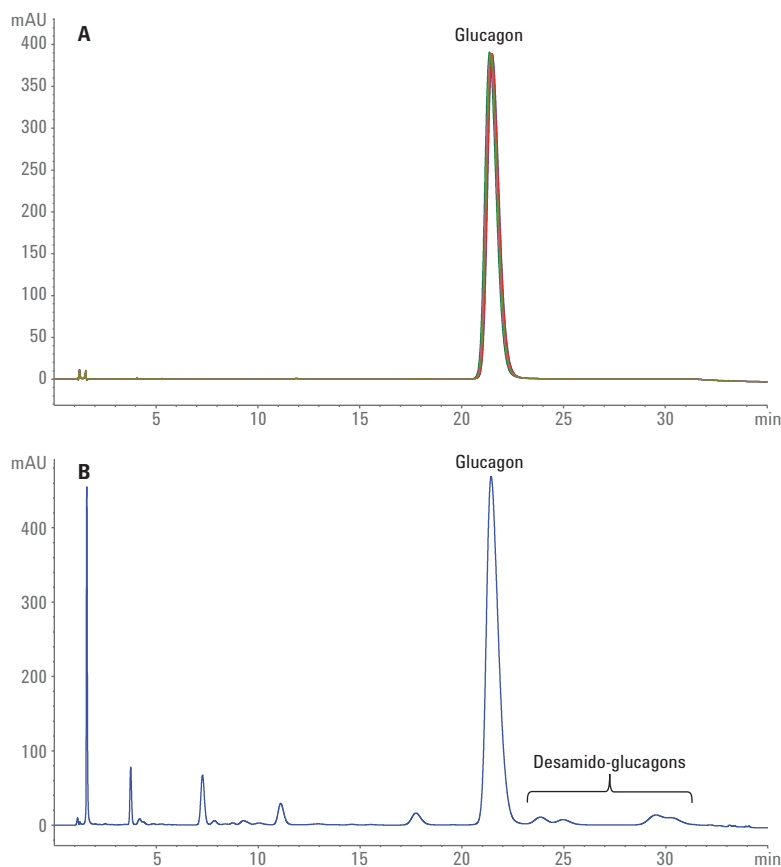


Figure 3. 1D chromatograms of the analysis of glucagon according to USP 39. A) Overlay of the fivefold analysis of the glucagon standard solution. B) Analysis of the glucagon system suitability solution.

Table 1. Suitability requirements according to USP 39 and results from the 1D analysis of the 2D-LC method.

Suitability requirements <sup>9</sup>	Results
Relative standard deviation (RSD): NMT 2.0 %, standard solution	Glucagon in standard solution (N = 5): RT RSD: 0.27 %, area RSD: 0.11 %
Tailing factor: NMT 1.8 for the glucagon peak, standard solution	Glucagon in standard solution: USP Tailing: 1.3
Resolution (Rs): Four peaks eluting after the glucagon peak that correspond to the desamido glucagons are clearly visible. The resolution between the main peak and the first eluting desamido peak is NLT 1.5, system suitability solution	System suitability solution: Four peaks eluting after the glucagon peak are clearly visible. Resolution between glucagon and first eluting desamido peak: 2.3

NMT = not more than  
NLT = not less than

Figure 4 shows the <sup>1</sup>D and <sup>2</sup>D chromatograms from the peak-based multiple heart-cutting 2D-LC analysis of the glucagon standard solution as displayed in the 2D-LC Viewer. Using a threshold of 5 mAU and a sampling time of 0.65 minutes, the peak apex of the <sup>1</sup>D glucagon peak was heart-cut and analyzed in the second dimension. In the <sup>2</sup>D MSD chromatogram, glucagon was detected after effective trapping on the AdvanceBio Desalting-RP cartridge. Figure 5 shows the corresponding mass spectrum of the glucagon peak. Glucagon was detected as the [M+5H]<sup>5+</sup>, [M+4H]<sup>4+</sup>, and [M+3H]<sup>3+</sup> ions. No adducts were detected, demonstrating the effectiveness of the desalting performed in the second dimension.

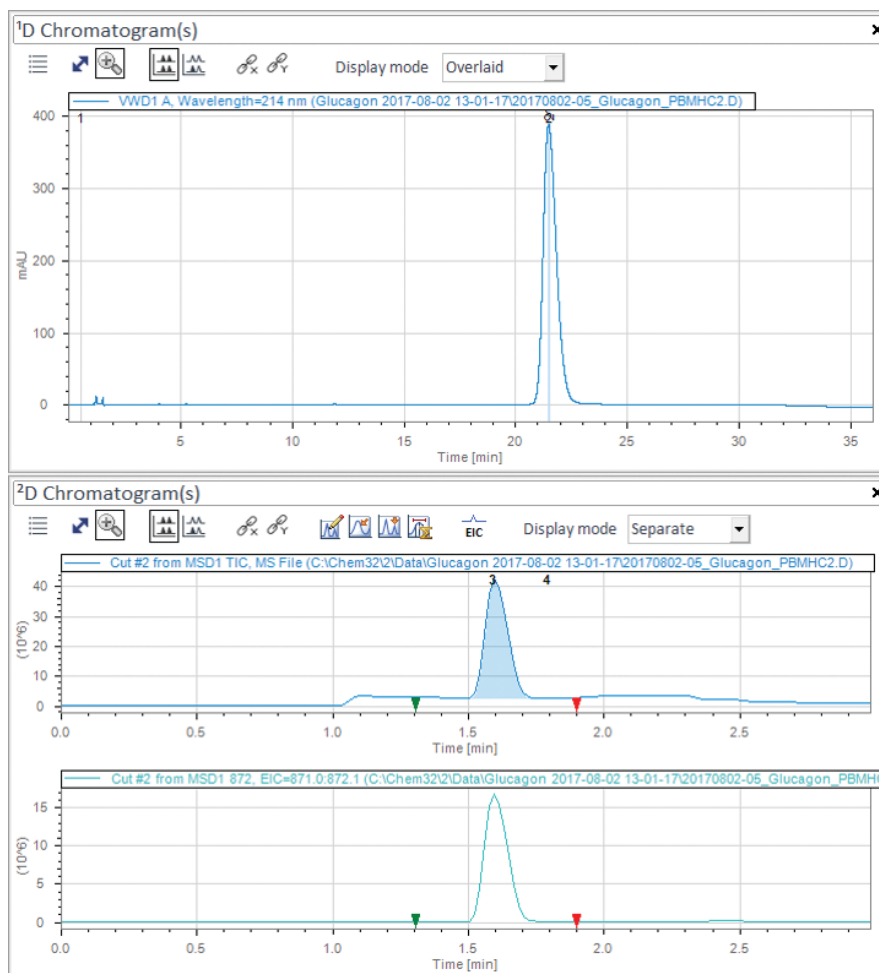


Figure 4. <sup>1</sup>D UV and <sup>2</sup>D MSD chromatograms (TIC and EIC of  $m/z$  871.0–872.1) of the peak-based multiple heart-cutting 2D-LC analysis of the glucagon standard solution.

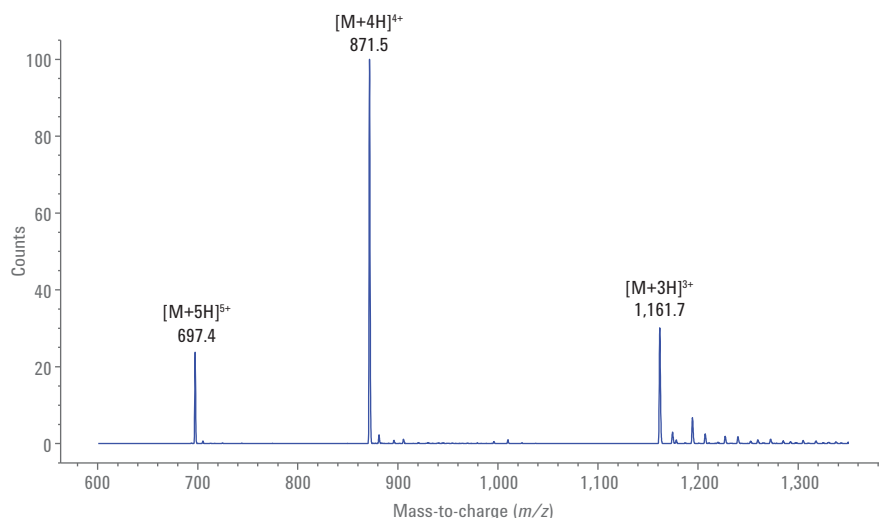


Figure 5. Mass spectrum of the glucagon peak.

For the glucagon system suitability solution, peak-based and time-based multiple heart-cutting 2D-LC analyses were performed. The aim of the peak-based multiple heart-cutting 2D-LC analysis was to heart-cut the glucagon peak as well as the peaks originating from the deamidation of glucagon to obtain mass information for these peaks. Using different sampling times for peak-based heart-cutting, peaks with different peak widths can be heart-cut around their peak apex. Figure 6A shows that the fourth desamido-glucagon peak was not heart-cut under these conditions, because it is not fully separated from the third desamido-glucagon peak. Using time-based multiple heart-cutting 2D-LC, peaks of interest such as the glucagon peak, the four peaks originating from the deamidation of glucagon, and further peaks originating from degradation products of glucagon can be heart-cut (Figure 6B). In this manner, mass information can be obtained for all target peaks after desalting in the second-dimension.

Figure 7 shows the mass spectrum of the first desamido-glucagon peak obtained after peak-based multiple heart-cutting 2D-LC analysis. The mass spectra of the four desamido-glucagon peaks are similar (data not shown). The mass increase observed for the  $[M+5H]^{5+}$ ,  $[M+4H]^{4+}$ , and  $[M+3H]^{3+}$  ions detected for these peaks, compared to the glucagon peak, confirms that these peaks originate from the deamidation of glucagon.

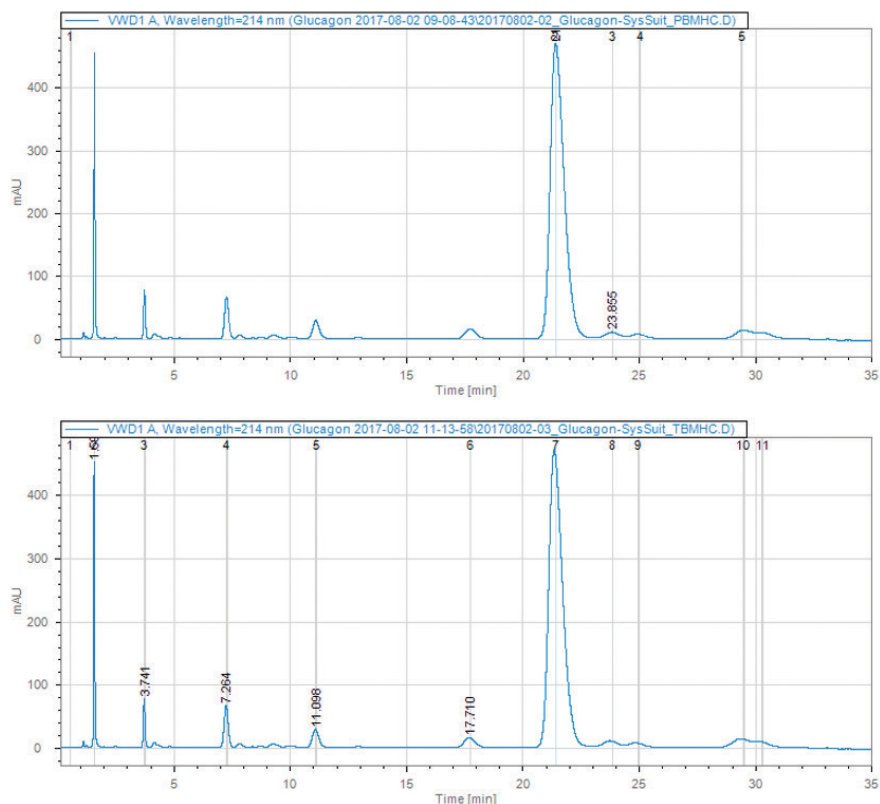


Figure 6. 1D chromatogram of the analysis of the glucagon system suitability solution with heart-cuts performed during (A) peak-based multiple heart-cutting 2D-LC and (B) time-based multiple heart-cutting 2D-LC.

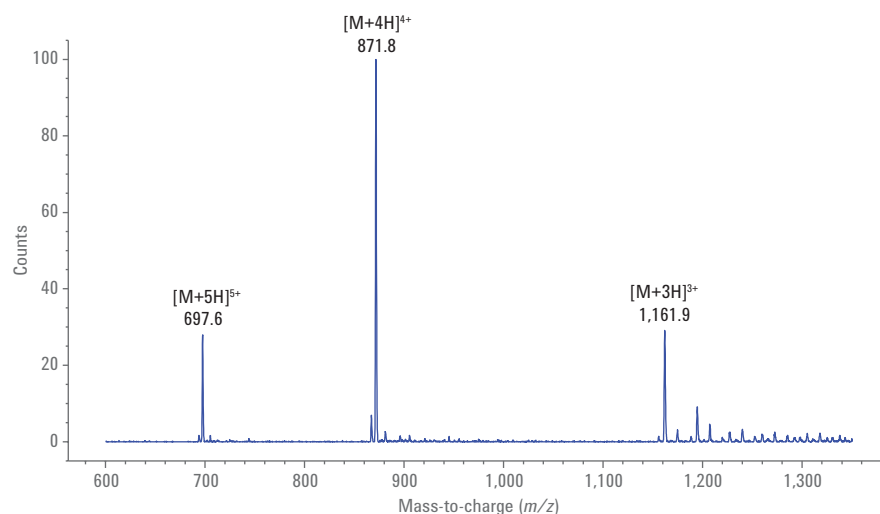


Figure 7. Mass spectrum of the first desamido-glucagon peak.



## Conclusion

Multiple heart-cutting 2D-LC with the Agilent InfinityLab 2D-LC Solution can be used as an effective desalting tool to allow online coupling of chromatographic methods using MS-incompatible mobile phases to MS detection. The analysis of the peptide glucagon according to USP 39 uses potassium phosphate buffer in the mobile phase. This MS-incompatible chromatographic method was coupled to mass selective detection after desalting in the second dimension. No adducts were detected, demonstrating the effectiveness of the desalting performed using Agilent AdvanceBio Desalting-RP cartridges. Mass information was obtained for target peaks of interest such as the glucagon peak and peaks resulting from deamidation of glucagon.

## References

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