High-Resolution Sampling 2D-LC for Pharmaceutical Impurity Analysis

Detection of Impurities Hidden Under the API Peak at Relevant Levels

Application Note

Small Molecule Pharmaceuticals and Generics

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Abstract

The analysis of impurities in low concentrations relative to an active pharmaceutical ingredient (API) is important for quality control of drug substances. When impurities are structurally similar to the API and the concentration difference is high, chromatographic separation and detection can be challenging.

The Agilent 1290 Infinity II 2D-LC Solution offers the possibility to switch easily between comprehensive (LCxLC), multiple heart-cutting (MHC), or high-resolution sampling 2D-LC (HiRes 2D-LC). In this Application Note, HiRes 2D-LC is used to achieve the separation of two closely eluting compounds, one of which is present at very low concentration, and is hidden under the other more highly concentrated compound peak. Chlorodifluorobenzoic acids and deamidated insulin were analyzed as standard substances and a real sample, respectively.

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Introduction

Purity analysis of drug substances is of high importance regarding quality and patient safety[1]. According to ICH Guideline Q3A(R2), an impurity in a new drug substance should be reported above a threshold of 0.05 %[2] relative to the API. Separation and detection of impurities at these levels can be challenging, especially when the impurity is structurally related to the active pharmaceutical ingredient (API).

This Application Note demonstrates the use of high-resolution (HiRes) sampling 2D-LC for the separation and detection of impurities, exemplified for different isomers of chlorodifluorobenzoic acid at relative concentrations of 100 % and 0.05 %. Precision and accuracy could further be improved for this application using the Agilent 1290 Infinity II High Dynamic Range Diode Array Detection (HDR-DAD) Impurity Analyzer Solution as the ^{2}D detector. Deamidated insulin is analyzed as a real sample to show the detection of a structurally similar impurity with HiRes sampling 2D-LC/MS. Here, 2D-LC has the additional benefit that the ^{2}D separation acts as a desalting step, avoiding ion suppression caused by high salt amounts from the ^{1}D buffer in the MS ion source[3]. The ^{2}D Chromatogram Creator for Agilent MassHunter is used for viewing and analyzing the 2D-LC/MS data in MassHunter.

In HiRes sampling 2D-LC, target compounds can be determined by collecting several small fractions over a selected time range, covering the entire area of a peak from the ^{1}D chromatogram, as shown in Figure 1. Each cut is parked in a sampling loop, and all cuts are analyzed in the second dimension consecutively. In this mode, it is ensured that all of the selected compound is transferred to, and analyzed in the second dimension. This way, selected coeluting compounds can be subjected to the HiRes sampling process for analysis in the second dimension. This enables a reliable quantification, as was shown in a Technical Overview[4].

Experimental

Equipment

The Agilent 1290 Infinity II 2D-LC Solution was composed of the following modules:

- Two Agilent 1290 Infinity II High Speed Pumps (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167B) with Infinity II Sample Cooler (Option #100)
- Two Agilent 1290 Infinity II Multicolumn Thermostats (G7116B)
- Three Agilent 1290 Infinity II Diode Array Detectors (G7117B) with 3.7-mm, 10-mm, and 60-mm Max-Light Cartridge Cells (G4212-60008)
- Agilent 1290 Infinity Valve Drive (G1170A) with 2-position/4-port-duo valve (2D-LC valve head, G4236A)
- Two Agilent 1290 Infinity Valve Drives (G1170A) with multiple heart-cutting valves (G4242-64000) equipped with 40-µL loops

For the analysis of deamidated insulin, the Agilent 1290 Infinity II 2D-LC Solution was coupled to an Agilent 6545 Q-TOF equipped with a Dual Agilent Jet Stream ESI source. To avoid high amounts of salt from the ^{1}D buffer entering the ion source, a timetable was used to switch the MS diverter valve, directing the flow to waste for the first minute of each ^{2}D analysis, as recommended in a previous Application Note[5].

Software

- Agilent OpenLAB CDS ChemStation Edition Rev. C.01.07 SR2 [255] with Agilent 2D-LC Software, Product Version A.01.03 [025], and Agilent HDR-DAD ChemStation AddOn, Product Version A.01.01 [015].
- Agilent MassHunter Workstation Software LC/MS Data Acquisition, Version B.08.00, Build 8.00.8026.0
- Agilent MassHunter Workstation Software Qualitative Analysis, Version B.07.00, Build 7.0.7024.0
- Agilent ^{2}D Chromatogram Creator for MassHunter, Rev. 1.0.15

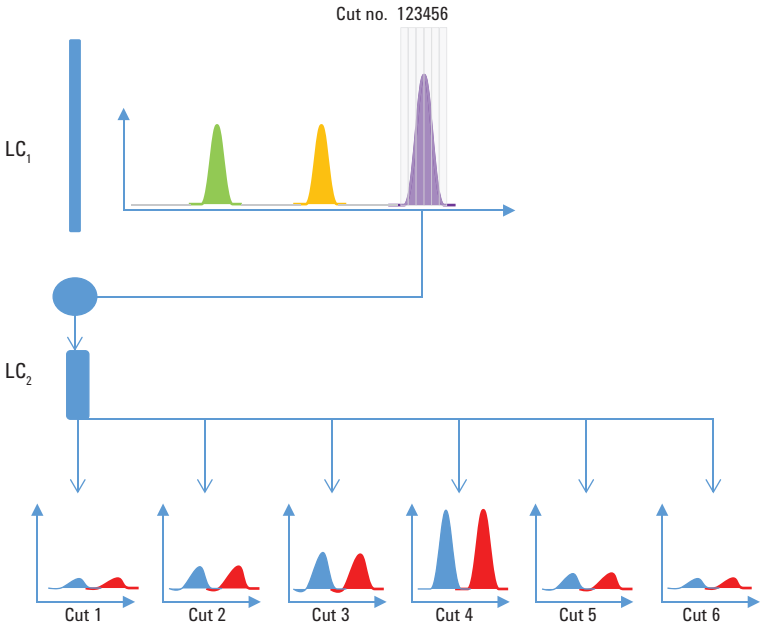


Figure 1. Illustration of high-resolution sampling 2D-LC.

Chemicals

All solvents were LC grade. Acetonitrile and sodium sulfate were purchased from Merck, Darmstadt, Germany. Formic acid, phosphoric acid, sodium phosphate dibasic, and ammonium phosphate monobasic were from Sigma-Aldrich, Steinheim, Germany. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22-µm membrane point-of-use cartridge (Millipak, EMD Millipore, Billerica, MA, USA). Standards of 5-chloro-2,4-difluorobenzoic acid, 3-chloro-2,4-difluorobenzoic acid, 2-chloro-4,5-difluorobenzoic acid, and insulin from bovine pancreas were purchased from Sigma-Aldrich, Steinheim, Germany.

Sample

Stock solutions of chlorodifluorobenzoic acids were prepared at a concentration of 1 mg/mL in 50/50 water/acetonitrile. Mixtures of the three compounds with different concentration ratios were prepared from these stock solutions.

A 1 mg/mL amount of insulin was dissolved in 25 mM sodium phosphate buffer at pH 2.55. To trigger deamidation, the insulin solution was adjusted to pH 9, and kept at room temperature for 12 hours.

High-resolution sampling 2D-LC method for the analysis of chlorodifluorobenzoic acids

	^{2}D Detector: DAD with 10-mm Max-Light cartridge cell	^{2}D Detector: HDR-DAD solution
Columns		
First dimension	Agilent ZORBAX Eclipse Plus C18 RRHD, 2.1 × 100 mm, 1.8 µm (p/n 959758-902)	
Second dimension	Agilent ZORBAX Eclipse Plus PAH, 2.1 × 100 mm, 1.8 µm (p/n 959764-918)	
^{1}D Pump		
Solvent A	Water + 0.1 % phosphoric acid	
Solvent B	Acetonitrile	
Flow rate	0.2 mL/min	
Gradient	0 minutes – 30 %B 10 minutes – 30 %B 12 minutes – 80 %B 14 minutes – 80 %B 15 minutes – 30 %B	
^{2}D Pump		
Solvent A	Water + 0.1 % phosphoric acid	
Solvent B	Acetonitrile	
Flow rate	0.5 mL/min	
Gradient	0 minutes – 20 %B 4 minutes – 25 %B 5 minutes – 25 %B	
^{2}D Gradient stop time	5.00 minutes	
^{2}D Cycle time	6.00 minutes	
Stop time	15 minutes	
High-resolution sampling		
Time based	6.54 minutes	7.83 minutes
Sampling time	7 seconds	9 seconds
Number of cuts	9	10
Multicolumn thermostat		
First dimension	30 °C	
Second dimension	30°C	
Multisampler		
Injection volume	1 µL	5 µL
Needle wash	10 seconds in methanol/water 50/50	
^{1}D Diode array detector		
Wavelength	210 nm/4 nm, reference 395 nm/10 nm	
Data rate	40 Hz	
^{2}D Detector		
	DAD (10 mm cell)	HDR-DAD
Wavelength	210 nm/4 nm, reference 395 nm/10 nm	
Data rate	40 Hz	

High-resolution sampling 2D-LC/MS method for the analysis of deamidated insulin (modified from)[3]

Parameter	Value
Columns	
First dimension	Agilent Poroshell 120 EC-C18 2.1 × 150 mm, 2.7 µm (p/n 693775-902)
Second dimension	Agilent ZORBAX Bonus RP 2.1 × 50 mm, 1.8 µm (p/n 857768-901)
^{1}D Pump	
Solvent A	60 mM Na_2SO_4 + 40 mM $NH_4H_2PO_4$, pH 2.21
Solvent B	Acetonitrile/water 80/20
Flow rate	0.3 mL/min
Gradient	0 minutes - 29 %B 28 minutes - 43 %B 29 minutes - 90 %B 30 minutes - 90 %B 31 minutes - 29 %B
^{2}D Pump	
Solvent A	Water + 0.1% formic acid
Solvent B	Acetonitrile/water 80/20 + 0.1% formic acid
Flow rate	0.4 mL/min
Gradient	0 minutes - 6.25% B 0.1 minutes - 23% B 2.6 minutes - 35% B 2.7 minutes - 90% B 2.8 minutes - 90% B 2.9 minutes - 6.25% B
^{2}D Gradient stop time	3.00 minutes
^{2}D Cycle time	5.00 minutes
Stop time	38 minutes
High-resolution sampling	
Time based	15.64 minutes
Sampling time	4 s
Number of cuts	10

Parameter	Value
Multicolumn thermostat	
First dimension	40 °C
Second dimension	40 °C
Multisampler	
Injection volume	1 µL
Needle wash	10 seconds in 50/50 methanol/water
^{1}D Diode array detector	
Wavelength	195 nm/4 nm, reference 395 nm/100 nm
Data rate	40 Hz
^{2}D Diode array detector	
Wavelength	195 nm/4 nm, reference 395 nm/100 nm
Data rate	40 Hz
MS parameters	
Mode	positive
Gas temperature	200 °C
Gas flow	13 L/min
Nebulizer	35 psig
Sheath gas temperature	375 °C
Sheath gas flow	12 L/min
VCap	2,500 V
Nozzle voltage	300 V
Fragmentor	175 V
Skimmer	65 V
Oct 1 RF Vpp	750 V
Mass range	100–3,200 *m/z*
Acquisition rate	2 spectra/s

MS diverter valve timetable

Time segment no.	Start time (min)	Diverter valve position
1	0	MS
2	16.02	Waste
3	17.02	MS
4	21.02	Waste
5	22.02	MS
6	26.02	Waste
7	27.02	MS
8	31.02	Waste
9	32.02	MS
10	36.02	Waste
11	37.02	MS
12	41.02	Waste

Time segment no.	Start time (min)	Diverter valve position
13	42.02	MS
14	46.02	Waste
15	47.02	MS
16	51.02	Waste
17	52.02	MS
18	56.02	Waste
19	57.02	MS
20	61.02	Waste
21	62.02	MS
22	66.02	Waste
23	67.02	MS

Method setup for high-resolution sampling 2D-LC

High-resolution sampling 2D-LC was performed with the Agilent 1290 Infinity II 2D-LC Solution. The valve configuration (Figure 2) consisted of a 2-position/4-port-duo valve connected to two multiple heart-cutting valves, holding 12 sampling loops. With this setup, up to 10 consecutive cuts can be sampled and stored until analysis. For HiRes sampling 2D-LC, a maximum loop filling of 80 % is recommended to prevent loss of sample. Figure 3 shows the method setup used for the ^{2}D pump for the analysis of chlorodifluorobenzoic acids. First, a 1D-LC separation of the sample was run, and the chromatogram was loaded as a reference signal in the preview window. HiRes sampling was set up time-based, according to the peak of interest, with nine cuts for the chlorodifluorobenzoic acids covering the entire peak width. Under the given ^{1}D conditions, a sampling time of 7 seconds equals a loop filling of 58 %. The higher injection volume applied with the Agilent 1290 Infinity II HDR-DAD Impurity Analyzer Solution as the ^{2}D detector required 10 cuts and a higher sampling time of 9 seconds to cover the entire ^{1}D peak width. This resulted in a loop filling of 75 %. For HiRes sampling of deamidated insulin, the method was set up following the same procedure. Ten cuts were taken with a sampling time of 4 seconds, which equals a loop filling of 50 %.

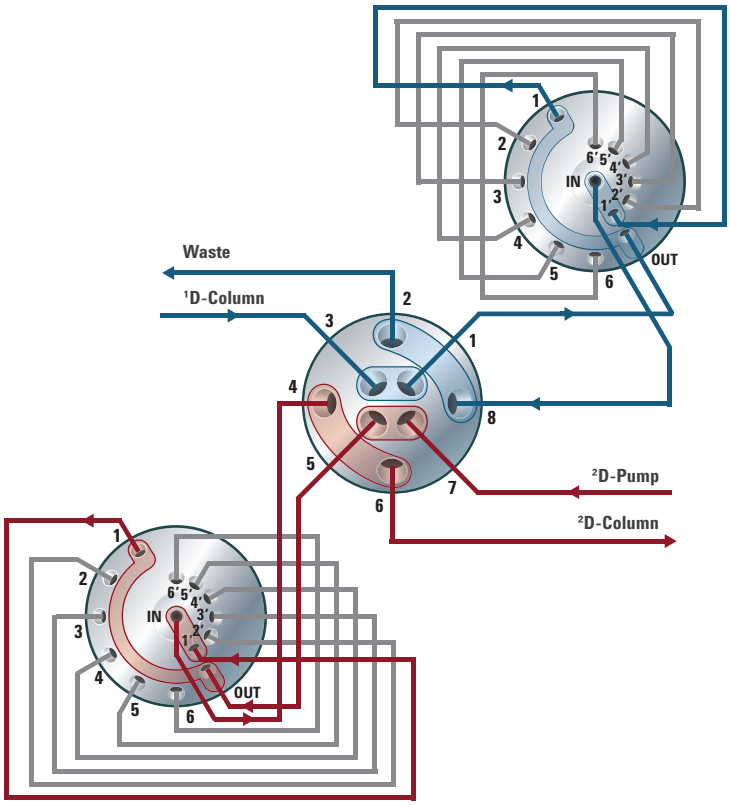


Figure 2. Valve configuration of the Agilent 1290 Infinity II 2D-LC Solution, holding 12 sampling loops.

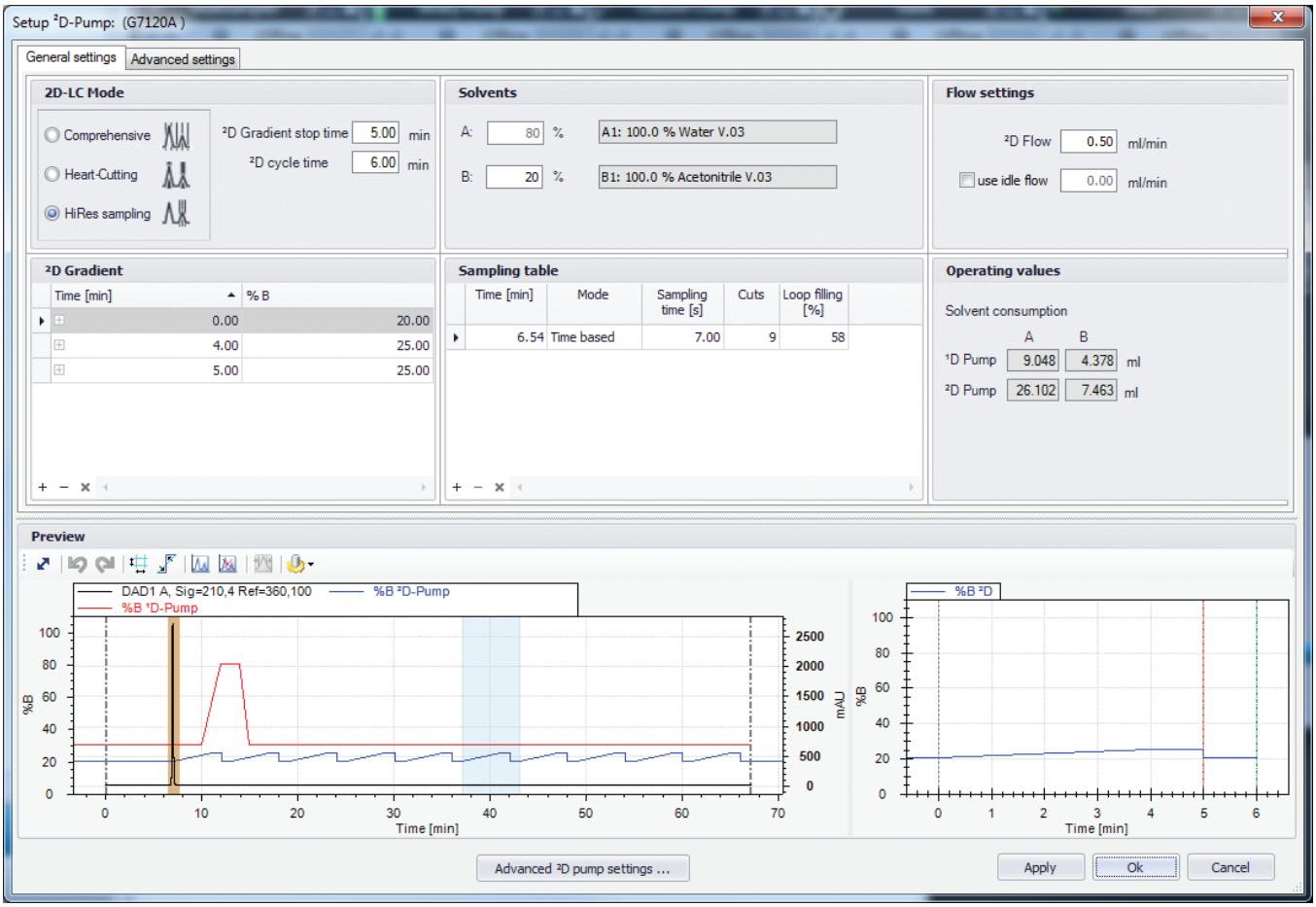
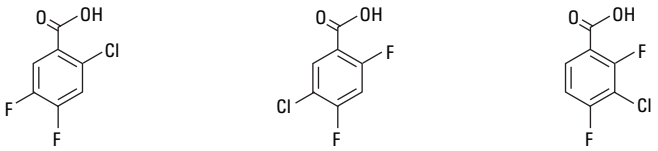


Figure 3. Method setup for the ²D pump.

Results and Discussion

Analysis of chlorodifluorobenzoic acids

Mixtures of the three isomers 2-chloro-4,5-difluorobenzoic acid (Compound 1), 5-chloro-2,4-difluorobenzoic acid (Compound 2) and 3-chloro-2,4-difluorobenzoic acid (Compound 3, structures in Figure 4) in different concentration ratios were analyzed using HiRes sampling 2D-LC, first with the Agilent 1290 Infinity II DAD with a 10-mm Max-Light cartridge cell as the ²D detector.



Compound 1
2-Chloro-4,5-difluorobenzoic acid

Compound 2
5-Chloro-2,4-difluorobenzoic acid

Compound 3
3-Chloro-2,4-difluorobenzoic acid

Figure 4. Chemical structures of the three analyzed chlorodifluorobenzoic acids.

Figure 5 shows the [1]D chromatograms of two mixtures containing all three compounds at the same low concentration level (blue), and in relative concentration levels of 100 % of Compound 3 and 0.05 % of Compounds 1 and 2, respectively (red). In the blue chromatogram, three separated peaks can be observed, whereas in the red chromatogram, Compound 2 (0.05 % relative concentration) is hidden underneath Compound 3 (100 % relative concentration), and could not be detected as a separate peak. In 2D-LC analysis of these compounds, HiRes sampling was used to sample the entire width of peak 3, followed by consecutive analysis of nine cuts on the [2]D column. Figure 6 shows the sampling scheme of the [1]D peak and the resulting [2]D chromatograms of Cuts 2–5. In Cut 2, no peak was detected.

Cut 3 shows two separated peaks of Compounds 2 and 3, which overlapped in the [1]D. Cut 4 contains mainly Compound 3 and a very small amount of Compound 2. In Cut 5, only Compound 3 was detected.

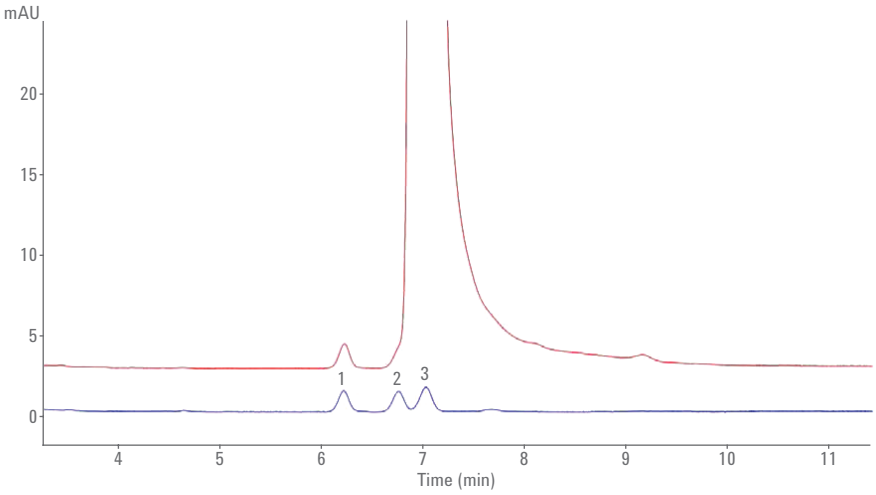


Figure 5. [1]D chromatograms of two mixtures in relative concentrations of 100 % Compound 3, 0.05 % Compounds 1 and 2 (red curve), and 0.05 % of all three compounds (blue curve).

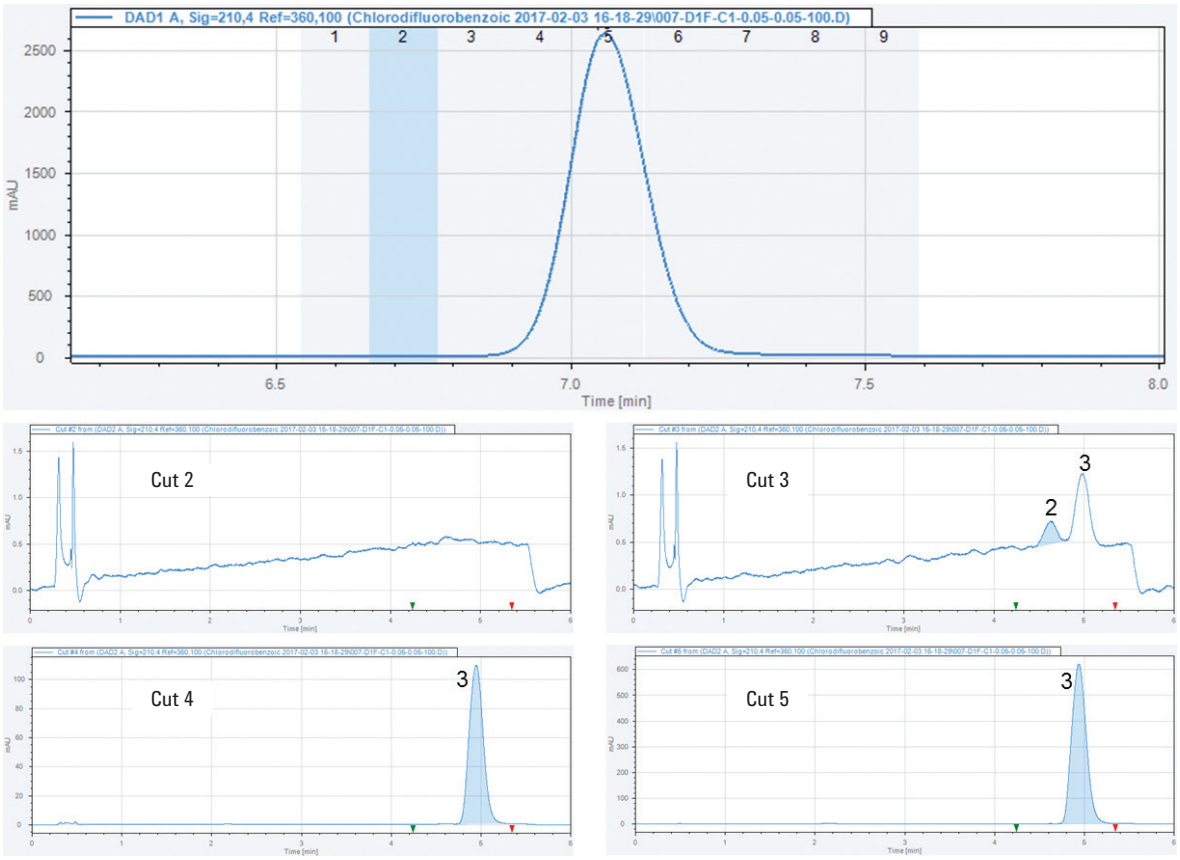


Figure 6. [1]D chromatograms with cuts 1–9 sampled over the entire peak width of Compound 3 (top). [2]D Chromatograms are shown for the analysis of Cuts 2, 3, 4, and 5.

To determine the usability of this method according to the reporting threshold given in the ICH guideline, different mixtures of the three chlorodifluorobenzoic acids were prepared, containing relative concentrations of 100 % of Compound 3 and 0.05 %, 0.10 %, or 0.15 % of Compounds 1 and 2. Peak areas of Compound 3 (3-chloro-2,4-difluorobenzoic acid) and Compound 2 (5-chloro-2,4-difluorobenzoic acid) were calculated as the sum of ^{2}D peaks in all cuts using the 2D-LC Viewer in Chemstation, as shown in Figure 7. Each mixture was analyzed in six replicates. Table 2 shows the average peak ares, as well as the relative standard deviations (RSDs) calculated for the three different mixtures. The peaks of the lower-concentrated Compound 2 have average signal-to-noise ratios (S/N) of 3.2, 5.9, and 12.2 for Mixes 1, 2, and 3, respectively, which is in the region of or below the limit of quantification (LOQ). This explains the relatively high RSD values of up to 7.95 % for the integrated peaks of Compound 2. Compound 2 could be detected in a relative area ratio of 0.03 %, 0.07 %, and 0.14 % compared to Compound 3. Considering a response factor of 1.17, which was determined from ^{1}D runs of both compounds, accuracy values of 71.6–108.5 % can be calculated, which is already a good result in light of the high concentration difference between the main compound and the impurity. This demonstrates that separation and detection of impurities in APIs at relevant levels according to the reporting threshold (0.05 %) given in ICH guideline Q3A(R2) is possible using high-resolution sampling 2D-LC for an impurity coeluting with the main peak in the first dimension.

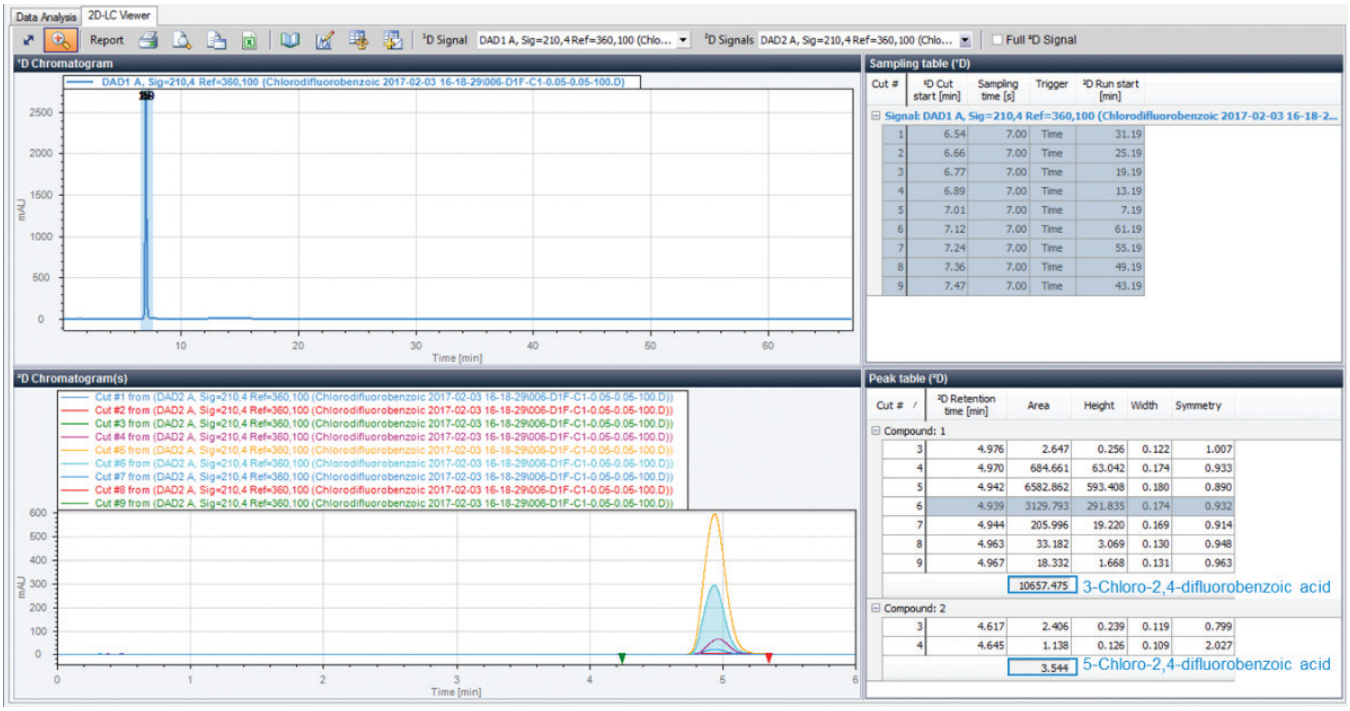


Figure 7. Results of the high-resolution sampling 2D-LC analysis of chlorodifluorobenzoic acids with peak areas of Compounds 2 (5-chloro-2,4-difluorobenzoic acid) and 3 (3-chloro-2,4-difluorobenzoic acid), as shown in the 2D-LC Viewer of Chemstation.

Table 2. Average peak areas and relative standard deviations calculated for three different mixtures from six consecutive runs with the Agilent 1290 Infinity II DAD with a 10-mm Max-Light cartridge cell as the ^{2}D detector. The area ratio of Compound 2 to Compound 3 is given in %. For calculation of accuracy, a response factor of 1.17, calculated from ^{1}D peaks, was considered.

	3-Chloro-2,4-difluorobenzoic acid (Compound 3)		5-Chloro-2,4-difluorobenzoic acid (Compound 2)			Area ratio (%)	Accuracy (%)
	Area	RSD (%)	Area	RSD (%)	S/N		
Mix 1 (0.05:0.05:100 %)	10,685.64	0.26	3.39	7.95	3.2	0.03	71.6
Mix 2 (0.10:0.10:100 %)	10,616.72	0.39	7.47	3.22	5.9	0.07	82.3
Mix 3 (0.15:0.15:100 %)	10,548.79	0.13	14.67	2.71	12.2	0.14	108.5

For further improvement of precision and accuracy, the Agilent 1290 Infinity II HDR-DAD Impurity Analyzer solution was used as the ^{2}D detector. This solution combines the signals from two diode array detectors with Max-Light cartridge cells of different path length to increase the linear dynamic UV range. This enabled a higher injection volume without exceeding the linear UV range for the high-concentrated Compound 3. Average S/N ratios were all above the LOQ, with 20.7, 24.1, and 34.0 for Mixes 1–3. The area precision of Compound 2 could significantly be improved, with RSD values below 1.58 % (Table 3). Accuracy was in the range of 96.4–108.5 %, which is excellent for quantification.

Analysis of deamidated insulin

Bovine insulin was deamidated under basic conditions to generate a real sample of a pharmaceutically relevant substance containing impurities.

The sample was analyzed with HiRes sampling 2D-LC/MS. The ^{2}D Chromatogram Creator for MassHunter combines measurement data from Agilent OpenLAB CDS ChemStation Edition and MassHunter. The software creates ^{2}D chromatograms, one chromatogram per cut in the first dimension, from the acquired full ^{2}D MS data. These chromatograms can be displayed in MassHunter similarly to what is offered by the 2D-LC viewer in OpenLAB CDS ChemStation Edition. The ^{1}D UV chromatogram, enriched by a signal indicating cuts, can be displayed in MassHunter, as shown in Figure 8A. Figure 8B shows the full 2D-MS, and Figure 8C the full 2D-UV

Table 3. Average peak areas and relative standard deviations calculated for three different mixtures from six consecutive runs with the Agilent 1290 Infinity II HDR-DAD Impurity Analyzer Solution as the ^{2}D detector. The area ratio of Compound 2 to Compound 3 is given in %. For calculation of accuracy, a response factor of 1.17, calculated from ^{1}D peaks, was considered.

	3-Chloro-2,4-difluorobenzoic acid (Compound 3)		5-Chloro-2,4-difluorobenzoic acid (Compound 2)				
	Area	RSD (%)	Area	RSD (%)	S/N	Area ratio (%)	Accuracy (%)
Mix 1 (0.05:0.05:100 %)	50072.70	1.19	23.30	1.58	20.7	0.05	108.5
Mix 2 (0.10:0.10:100 %)	49020.63	0.85	42.02	1.44	24.1	0.09	99.9
Mix 3 (0.15:0.15:100 %)	48698.74	1.34	60.37	1.47	34.0	0.12	96.4

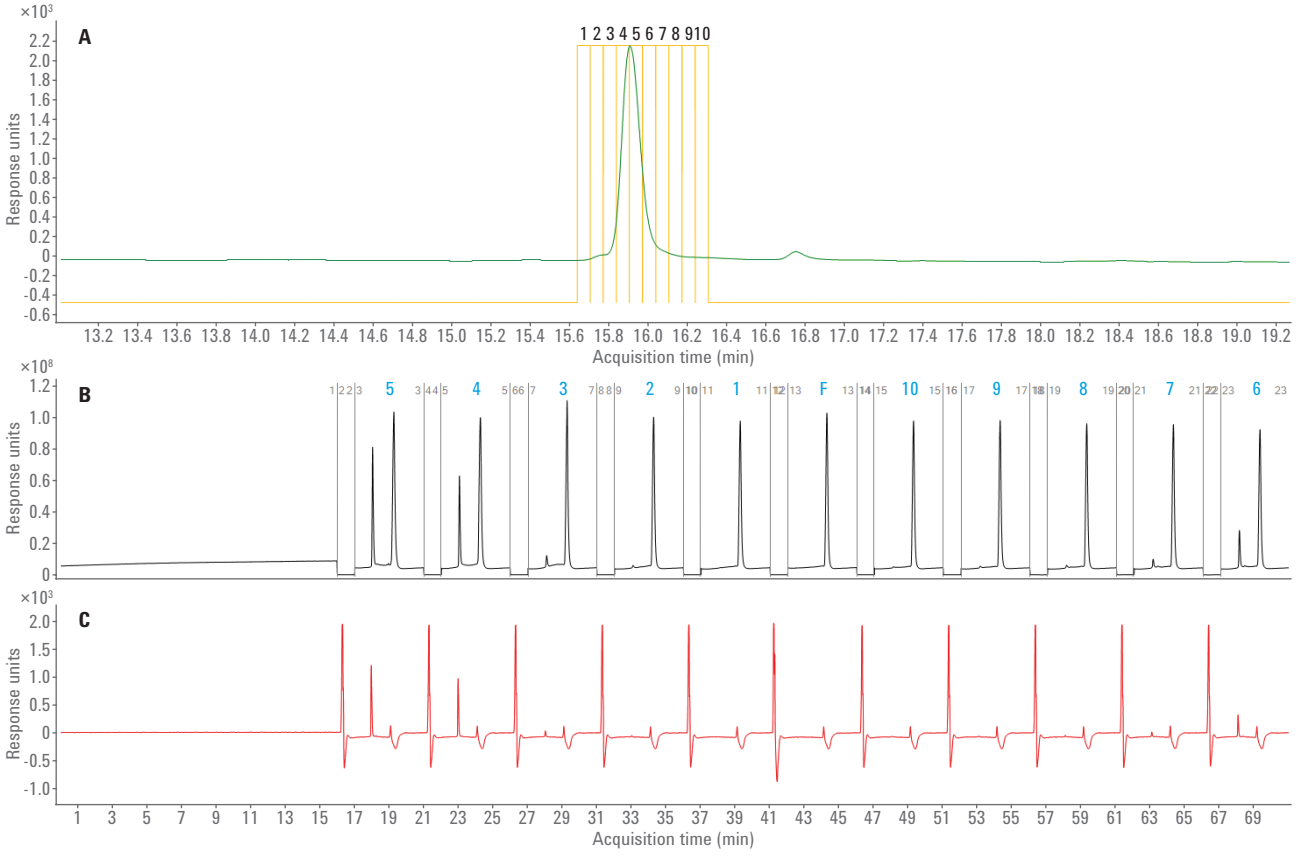


Figure 8. Signals displayed in Agilent MassHunter for the analysis of deamidated insulin. A) ^{1}D-UV signal with cuts taken in HiRes sampling. B) Full ^{2}D-MS signal. C) Full ^{2}D-UV signal.

signal in MassHunter. For each of the 10 cuts, one individual chromatogram was created using the ^{2}D Chromatogram Creator for MassHunter. These total ion chromatograms (TICs) are stacked for all 10 cuts in Figure 9. During the first minute of each ^{2}D separation, the flow was directed to waste by switching the MS diverter valve. Buffer salts contained in the fractions were excluded from the MS ion source in this manner. In each TIC in Figure 9, a step is visible at 1 minute run time when the diverter valve is switched back, and the flow is directed to the MS. The main insulin peak can be observed with a retention time of 2.06 minutes at different intensities in Cuts 2–10. The ^{2}D gradient includes a short section with 90 %B for flushing the column, leading to a rapid increase and decrease of the baseline at approximately 3.2 minutes. Figure 10 shows a closer view of the TIC and the corresponding ^{2}D-UV signal for Cut 7, which was taken from the first dimension in the tailing of the main insulin peak. In the ^{2}D chromatogram, two peaks can be observed in both the MS and the UV signal. Figure 11 shows that the mass spectra of both were extracted. The isotopic pattern of peak 1 can be referred to as the $[M+5H]^{5+}$ ion of insulin. The mass spectrum of peak 2 shows an isotopic pattern corresponding to a single deamidation product of insulin (neutral mass shift of 0.98 Da). The same compound could be detected in Cut 6 (data not shown).

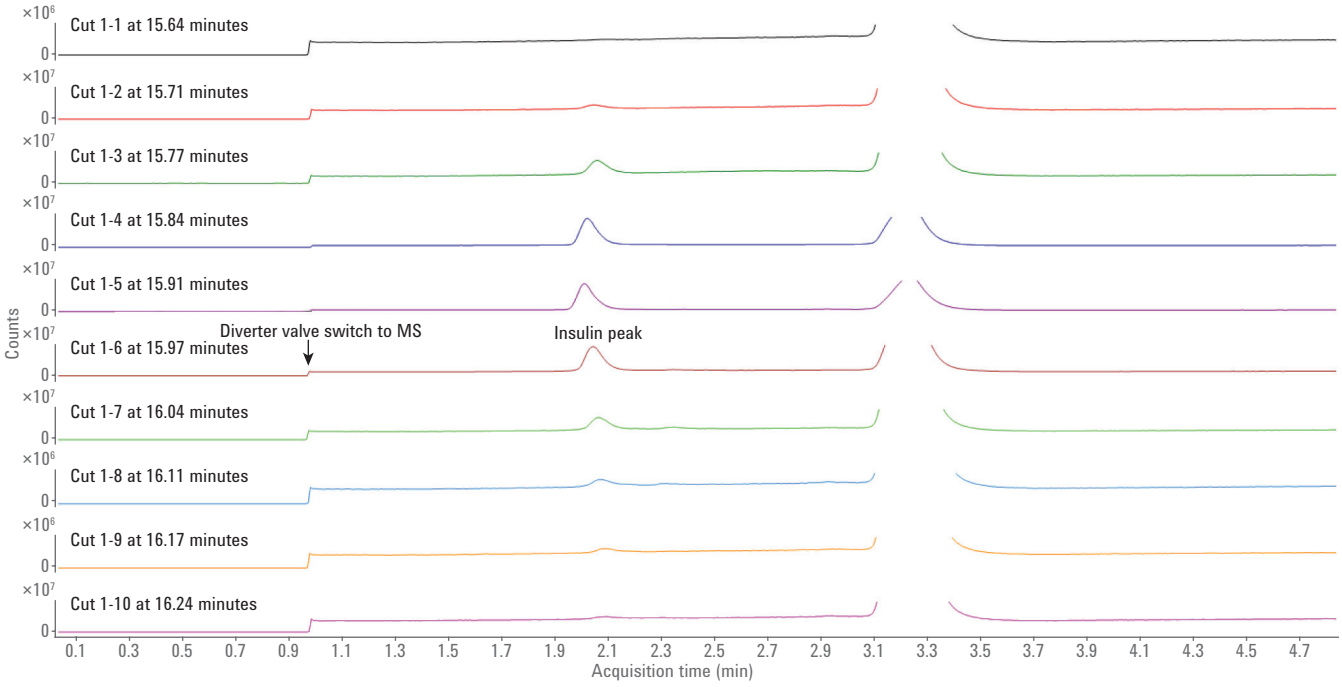


Figure 9. Individual ^{2}D chromatograms of all cuts generated with the ^{2}D Chromatogram Creator for Agilent MassHunter.

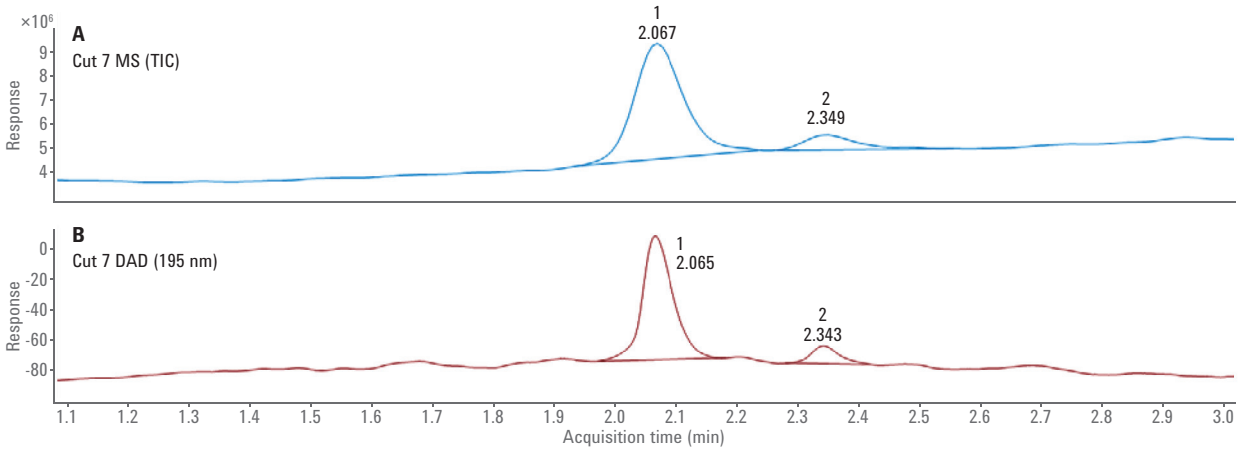


Figure 10. [2]D chromatogram of Cut 7, generated from the MS (TIC) and the UV signal (DAD2).

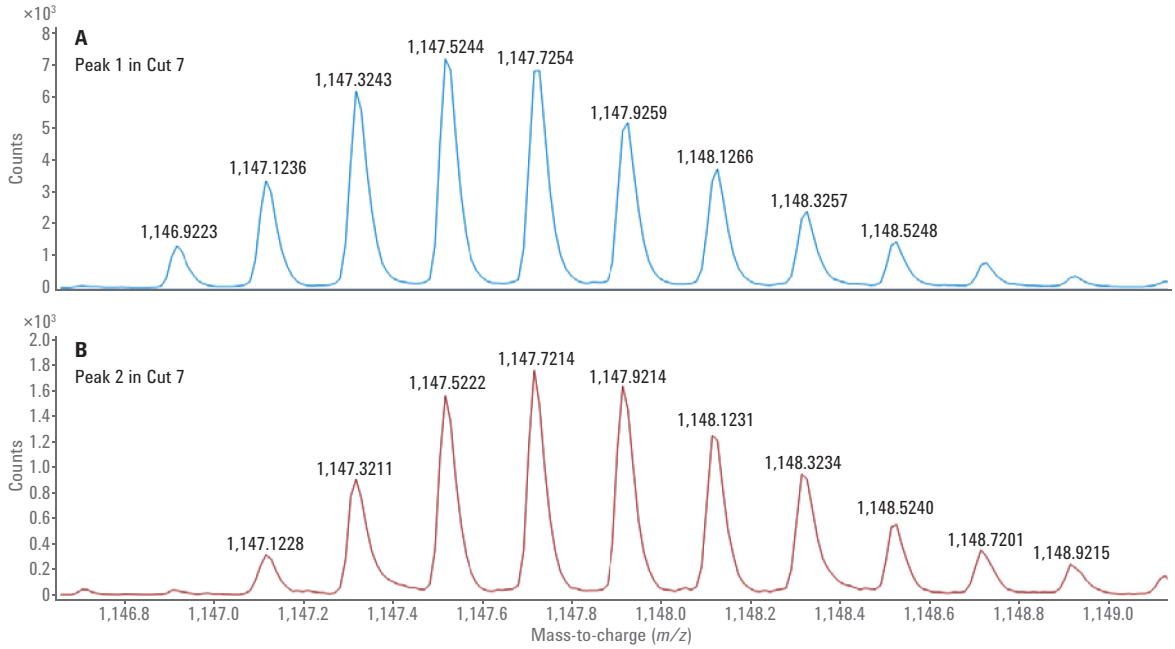


Figure 11. Mass spectra of peaks 1 and 2 observed in the [2]D chromatogram of Cut 7.

Conclusions

This Application Note demonstrates the detection of low-level impurities contained in a sample of a structurally related main compound using high-resolution sampling 2D-LC. Using a mixture of chlorodifluorobenzoic acids, it is shown that compounds at relative concentrations of 0.05 %, 0.10 %, 0.15 %, and 100 % according to the ICH Guideline about impurities in new drug substances, which cannot be detected separately in ^{1}D analysis, are transferred to and separated on the ^{2}D column by HiRes sampling 2D-LC. The compound 5-chloro-2,4-difluorobenzoic acid was detected with accuracy values of 71.6–108.5 %. Area precision was 2.71–7.95 %. The Agilent 1290 Infinity II HDR-DAD Impurity Analyzer Solution as the ^{2}D detector allowed a higher injection volume, resulting in improved accuracy of 96.4–108.5 % and area precision of 1.44–1.58 %. Deamidated insulin was used as a real sample for analysis with HiRes sampling 2D-LC/MS. Data were evaluated using the ^{2}D Chromatogram Creator for Agilent MassHunter. A single deamidation product of insulin was detected and identified through its isotopic pattern in two cuts, which were taken from the first dimension in the tailing of the main insulin peak.

References

1. Venkatramani, C. J.; *et al.* Assessing Stability-Indicating Methods for Coelution by Two-Dimensional Liquid Chromatography with Mass Spectrometric Detection. *J. Sep. Sci.* **2014**, *37*, 3214–3225.

2. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use – Impurities in New Drug Substances Q3A(R2), **2006**.

3. Petersson, P.; Haselmann, K.; Buckenmaier, S. Multiple Heart-Cutting Two Dimensional Liquid Chromatography Mass Spectrometry: Towards Real Time Determination of Related Impurities of Bio-Pharmaceuticals in Salt Based Separation Methods. *J. Chromatogr. A* **2016**, *1468*, 95–101.

4. Stephan, S. High-Resolution Sampling 2D-LC with the Agilent 1290 Infinity II 2D-LC Solution. *Agilent Technologies Technical Overview*, publication number 5991-7637EN, **2016**.

5. Schneider, S. 2D-LC/MS Characterization of Charge Variants Using Ion Exchange and Reversed-Phase Chromatography Multiple Heart-Cutting 2D-LC Analysis of Innovator versus Biosimilar Monoclonal Antibodies. *Agilent Technologies Application Note*, publication number 5991-6673EN, **2016**.

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