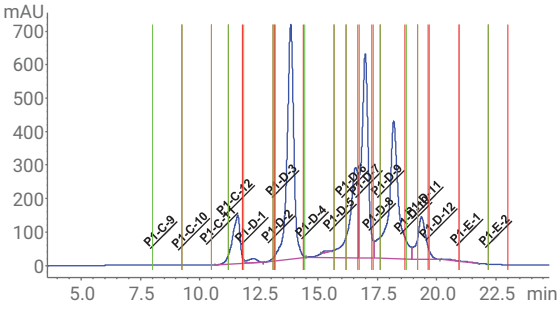
Application Note

Biologics & Biosimilars

Fraction Analysis of Cysteine-Linked Antibody-Drug Conjugates Using Hydrophobic Interaction Chromatography

Agilent 1260 Infinity II Bio-Inert System



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Abstract

This Application Note describes the peak-based fraction collection of the cysteine-linked antibody-drug conjugate (ADC) brentuximab vedotin after hydrophobic interaction chromatography (HIC). The combination of the Agilent 1260 Infinity II Bio-Inert LC with the Agilent 1260 Infinity II Bio-Inert Fraction Collector enabled fraction collection as well as re-analysis of biopharmaceuticals in a complete metal-free flow path while preventing corrosion caused by the high salt containing buffers used in HIC. The improved features of the 1260 Infinity II Bio-Inert Fraction Collector achieved highly precise fraction collection as proved by re-analysis using the same HIC method. The fractionation as well as the identification of the ADC main peaks was confirmed with re-analysis using reversed-phase chromatography.

Introduction

The availability of possible conjugation sites means that cysteine-linked antibody-drug conjugates (ADCs) are typically less heterogenous regarding the distribution and quantity of conjugated small molecule drugs when compared to lysine-linked ADCs. Different numbers of drugs can be conjugated to the monoclonal antibody (mAb) in the conjugation process, Therefore, cysteine-linked ADCs contain a mixture of isoforms with 0, 2, 4, 6, and 8 drugs attached. In addition to the drug-to-antibody ratio (DAR), the analysis of the drug distribution is essential to ensure the safety and efficacy of the drug. The reduced complexity enables analysis using high performance liquid chromatography (HPLC) analytical methods with hydrophobic interaction chromatography (HIC), for example, to characterize the heterogeneity of the ADC. The analysis of ADCs using HIC is described in a previous Application Note[1]. The information provided by HIC can be insufficient due to a different distribution profile than the expected profile with five peaks, representing the DAR values of 0, 2, 4 ,6, and 8. An additional analysis might be required.

The automation of biomolecule semi-preparative workflows represents major challenges in analytical liquid chromatography. Low flow rates and time-based fraction collection lead to considerable limitations in small-scale preparation. Time-based fractionation is prone to errors due to mistimed fractionation points, resulting in potential split fractions in a single peak.

Especially for HIC workflows, where an extremely high amount of salt is used (ammonium sulfate in a concentration of up to 2 M in mobile phase A), the Agilent 1260 Infinity II Bio-Inert LC System is an excellent fit to prevent corrosion, which is often found in stainless steel systems.

The 1260 Infinity II Bio-Inert LC in combination with the Agilent 1260 Infinity II Bio-Inert Fraction Collector allows the user to perform highly accurate peak-based fraction collection in a completely metal-free flow path. In addition, the 1260 Infinity II Bio-Inert Fraction Collector contains improved features to raise peak-based fractionation to a new level.

This Application Note describes the HIC analysis of brentuximab vedotin, fraction collection of the main peaks, and re-analysis using HIC and reversed-phase chromatography.

Experimental

Instrumentation

The Agilent 1260 Infinity II Bio-Inert LC system consisted of the following modules:

- Agilent 1260 Infinity II Bio-Inert Pump (G5654A)
- Agilent 1260 Infinity II Bio-Inert Multisampler (G5668A) with sample cooler (Option 100)
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A) with bio-inert heat exchanger (Option 019)
- Agilent 1260 Infinity II Diode Array Detector WR (G7115A) with bio-inert flow cell (Option 028)
- Agilent 1260 Infinity II Bio-inert Fraction Collector (G5664B)

Columns

- Generic HIC column
- Agilent PLRP-S 300Å, 2.1 × 50 mm, 3 µm HPLC column (PL1912-1301)

Software

Agilent OpenLAB CDS ChemStation Edition for LC Systems, version C.01.07 SR3

Sample

Brentuximab vedotin (Adcetris) dissolved in Buffer B at 200 mg/mL

Sample Preparation After Fractionation

The HIC fractions and the pure ADC were reduced with 30 mM *tris*(2-carboxyethyl) phosphine (TCEP) at 37 °C for 1 hour before re-analysis by reversed-phase chromatography.

Chemicals

Trifluoroacetic acid (TFA), sodium phosphate monobasic and dibasic, ammonium sulfate, and *tris*(2-carboxyethyl)phosphin were purchased from Sigma-Aldrich, St. Louis, Missouri, USA. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with an LC-Pak Polisher and a 0.22-µm membrane point-of-use cartridge (Millipak). All solvents used were LC grade. Acetonitrile and isopropanol were purchased from Merck, Germany.

Results and Discussion

Brentuximab vedotin was analyzed using HIC, revealing five main peaks that correspond to the mAb containing 0, 2, 4, 6, or 8 MMAE drugs (see Figure 1). The peaks were identified by comparing the HIC chromatogram to those for brentuximab vedotin found in literature[1,2].

Table 1. Chromatographic conditions for HIC analysis for fractionation and re-analysis.

	Chromatographic conditions
Mobile phase	A) 2 M ammonium sulfate in 100 mM sodium phosphate pH 7 B) 100 mM sodium phosphate pH 7 C) Isopropanol
Flow rate	0.4 mL/min
Gradient	0 minutes – 55 %A, 45 %B, 0 %C 15 minutes – 0 %A, 80 %B, 20 %C 20 minutes – 0 %A, 80 %B, 20 %C 21 minutes – 55 %A, 45 %B, 0 %C
Stop time	31 minutes
Needle wash mode	Standard Wash
Injection volume	20 µL
Column temperature	30 °C
Diode array detection	280 nm/4 nm >0.025 minutes (0.5 seconds response time) (10 Hz)

Table 2. Chromatographic conditions for reversed-phase re-analysis of HIC fractions.

	Chromatographic conditions
Mobile phase	A) Water + 0.1 % TFA B) ACN + 0.1 % TFA
Flow rate	0.3 mL/min
Gradient	0 minutes – 75 %A, 25 %B 1 minutes – 60 %A, 40 %B 15 minutes – 50 %A, 50 %B 16 minutes – 20 %A, 80 %B
Stop time	16 minutes
Post time	5 minutes
Needle wash mode	Standard Wash
Injection volume	30 µL
Column temperature	90 °C
Diode array detection	280 nm/4 nm >0.025 minutes (0.5 seconds response time) (10 Hz)

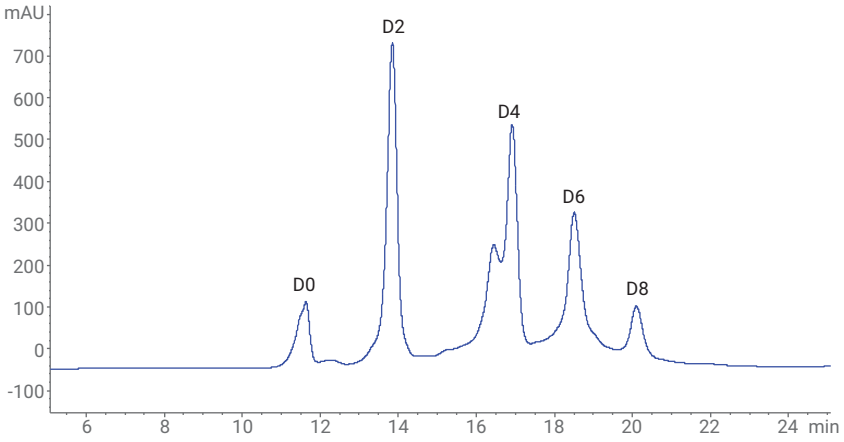

Figure 1. Analysis of brentuximab vedotin on the Agilent 1260 Infinity II Bio-inert LC. D0–D8 refers to different DAR species.

To enable further analysis, the main peaks were collected using the 1260 Infinity II Bio-Inert Fraction Collector. To find the optimal collection parameters, the HIC chromatogram of brentuximab vedotin was loaded in the method settings of Agilent OpenLAB CDS ChemStation Edition in the Fraction Preview option. Figure 2 displays the fraction collection method setup with the loaded HIC chromatogram. Based on the previously recorded chromatogram, the following fraction options were chosen:

Peak-based fractionation was chosen as the fraction trigger mode. With this mode, the user can estimate and enter basic parameters for peak threshold and slope based on experience and the expected sample concentration. A combination of threshold and slope was chosen for peak detection, whereas the thresholds (three different thresholds were chosen), as well as the slopes, were directly determined in the previously recorded chromatogram in the fraction preview.

Volume slice recovery fraction mode: Whenever the signal rises above the entered threshold or slope parameters, the fraction mode switches to peak-based, and collects distinct fractions depending on the signal. With this option, the compounds of interest are collected in single fractions, whereas the volume slice recovery collection prevents any sample loss in case threshold/slope parameters are improperly set. Fraction collection starts with volume slice recovery at the time specified in the timetable.

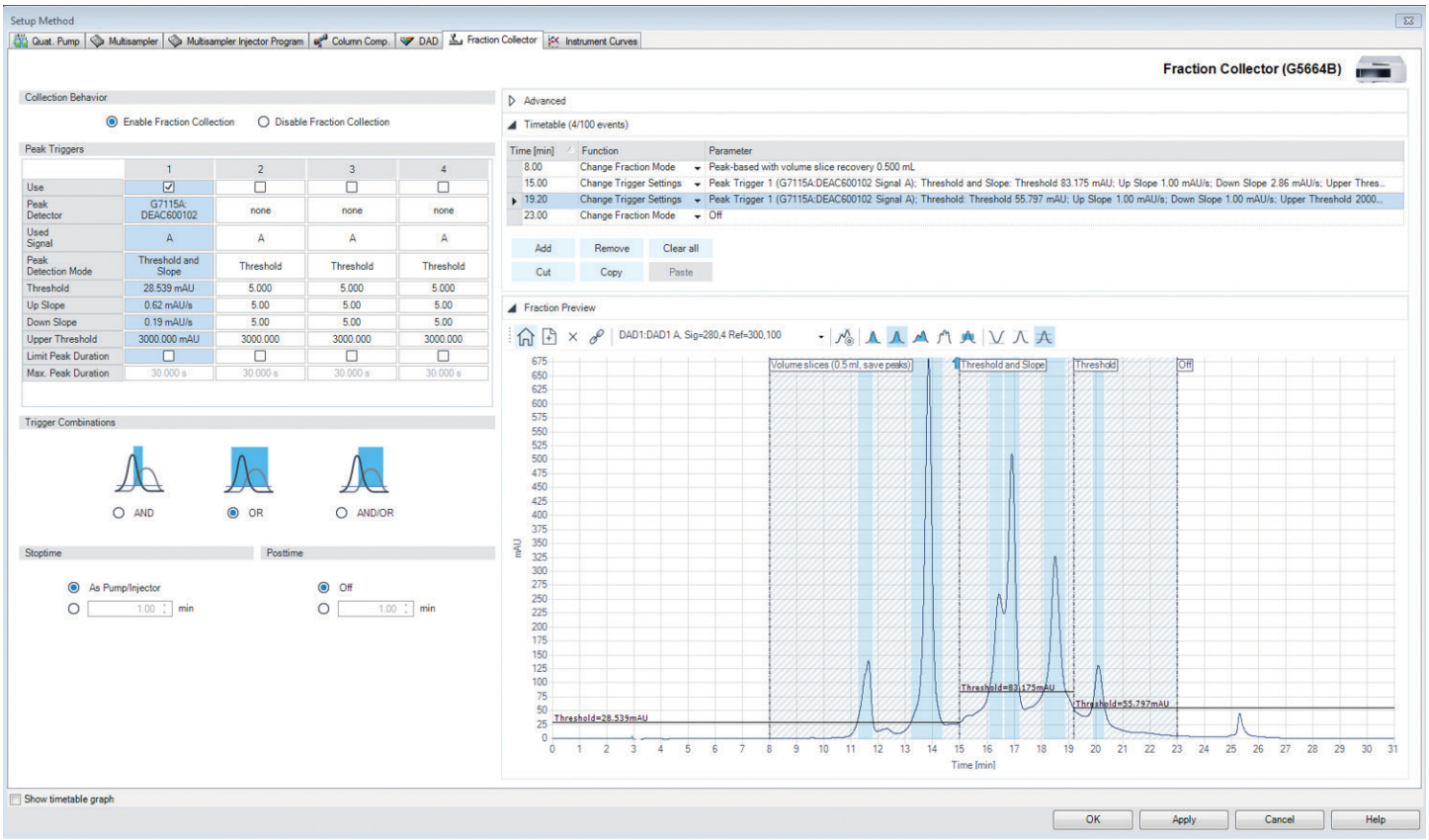

Figure 2. Fraction collector method settings in Agilent OpenLAB CDS ChemStation Edition, displaying collection timetable, peak trigger modes, and fraction preview. The latter enables the user to review how changes of threshold, slope, and time settings affect the fraction collection.

Figure 3 shows the resulting chromatogram displaying the collected fractions in the data analysis.

To confirm successful fraction collection, the fractions were re-analyzed using the same HIC method that was used for the separation of brentuximab vedotin but disabling fraction collection in the method setup (Figure 2 shows this option). Figure 4 shows the re-analysis using HIC for brentuximab vedotin as well as the collected main peaks. The re-analysis using HIC demonstrates successful fractionation.

To confirm the identification found in literature, additional analysis was required. We used a reversed-phase analytical method for additional confirmation. Reversed-phase HPLC can be applied to separate and qualify light- and heavy-chain species carrying different drug loads. Figure 5 shows a separation of TCEP-reduced brentuximab vedotin, enabling a determination of light chain (LC), light chain plus one small molecule drug load (LC+1), heavy chain (HC), and heavy chain plus one, two, and three drug loads (HC+1, HC+2, HC+3) compared to literature[3].

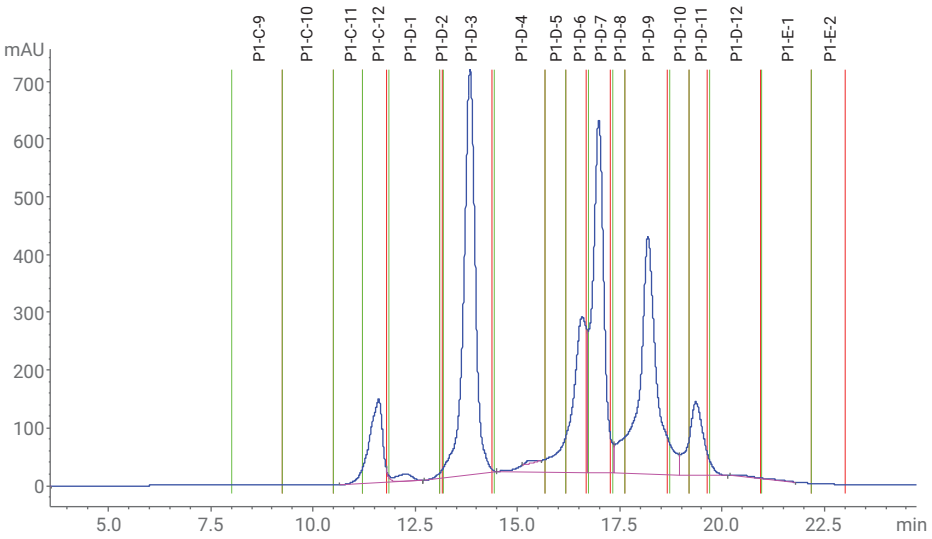

Figure 3. Collected fraction after HIC with a combination of peak-based fraction detection and volume slice recover fraction collection mode.

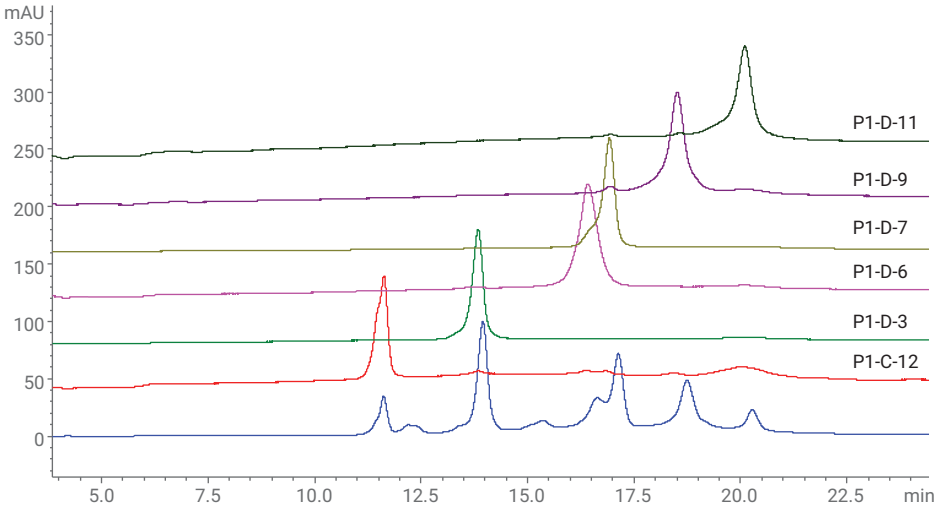

Figure 4. Overlay of the HIC analysis of brentuximab vedotin in blue (lowest chromatogram) as well as the collected fractions.

Figure 6 shows the re-analysis of the collected and reduced HIC fractions using reversed-phase LC with the Agilent PLRP-S column, which is specially suited for very hydrophobic samples such as ADCs. The re-analysis of the fractions is shown in the small chromatograms below the HIC fractionation. The reversed-phase analysis of the single fractions is in complete accordance with the HIC separations. The first peak in the HIC chromatogram (D0, fraction C12) contains LC and HC with no drug load, confirmed by the two main peaks representing LC and HC in the reversed-phase chromatogram. The re-analysis of the second peak (D2, fraction D3) contains four main peaks, representing LC, LC+1, HC, and HC+1. The re-analysis of the third peak (D4, fraction D7) contains five main peaks, representing LC, LC+1, HC, HC+1, and HC+2. The re-analysis of the fourth peak (D6, fraction D9) contains LC, LC+1, HC+2, HC+3, enabling a total drug load of six small molecules. In the re-analysis of D8 (fraction D11), there are only two main peaks visible: LC+1 and HC+3, which makes a drug load of eight small molecules. In addition to the re-analysis using HIC, the re-analysis using reversed-phase analysis confirms not only the correct fractionation after the HIC separation of brentuximab vedotin, but also the correct peak labeling in the HIC chromatogram.

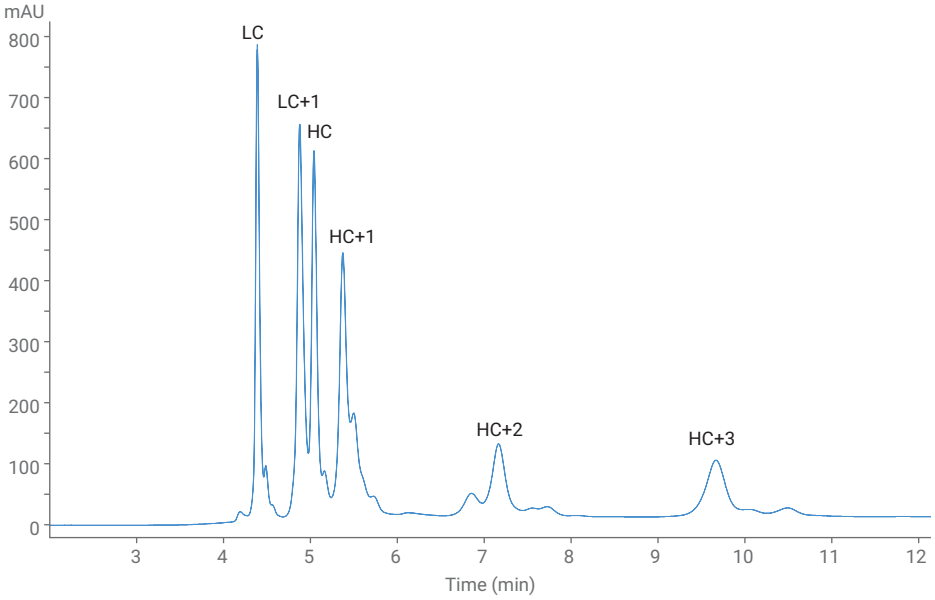

Figure 5. Separation of Brentuximab vedotin using the Agilent PLRP-S column.

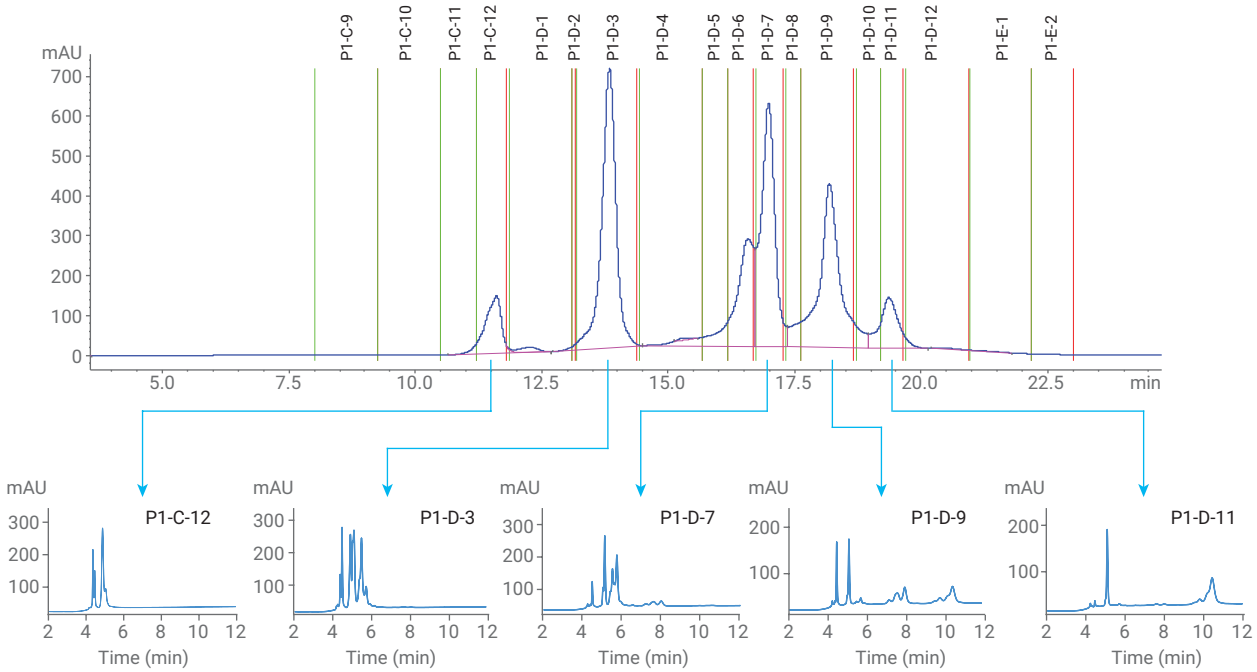

Figure 6. Fraction collection of Brentuximab vedotin after HIC and re-analysis using reversed-phase with Agilent PLRP-S column.

Conclusion

Peak-based fraction collection was performed after the HIC separation of brentuximab vedotin. The peak-based fraction trigger mode was enhanced with improved features for optimal and exact fraction collection. The correct fractionation as well as the identification of the ADC main peaks was confirmed using HIC and reversed-phase re-analysis.

The Agilent 1260 Infinity II Bio-Inert LC with the Agilent 1260 Infinity II Bio-Inert Fraction Collector was shown to be an optimal combination for the fractionation and re-analysis of ADCs.

References

1. Schneider, S. Analysis of Cysteine-linked Antibody Drug Conjugates using Hydrophobic Interaction Chromatography on the Agilent 1260 Infinity II Bio-Inert LC, *Agilent Technologies Application Note*, publication number 5991-8493EN, **2017**.
2. Younes; *et al.* Brentuximab Vedotin (SGN-35) for Relapsed CD30-Positive Lymphomas, *N. Engl. J. Med.* **2010**, *363*, 1812–1821.
3. Wakankar, A.; *et al.* Analytical methods for physiochemical characterization of antibody drug conjugates, *Landes BioScience*, *mAbs* **2011**, *3:2*, 161–172.

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