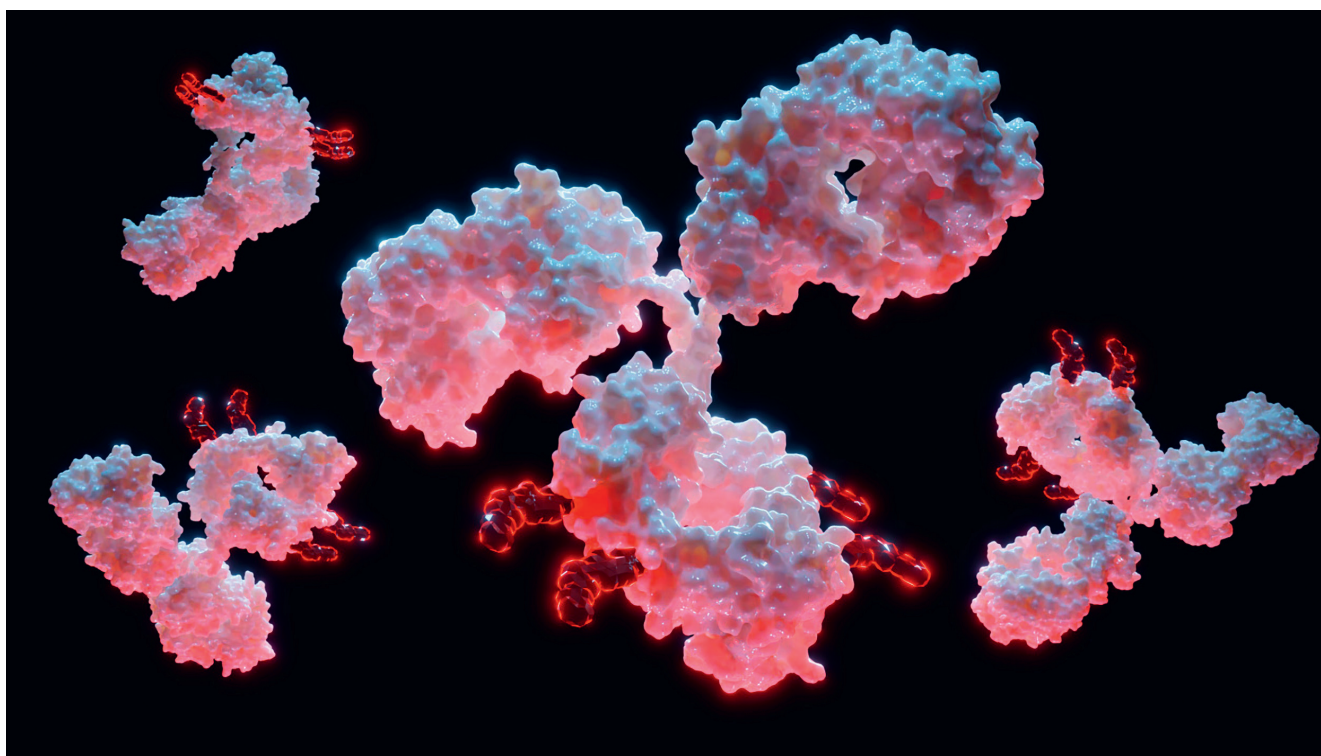


## DAR determination of a 3<sup>rd</sup> generation, site-specific ADC using reversed phase chromatography

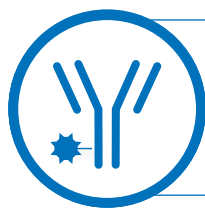
Antibody-drug conjugates (ADCs) represent a rapidly evolving class of targeted therapies, with three distinct generations of conjugation methods. The first generation utilises lysine residues for drug attachment, while the second generation relies on cysteine coupling.

The third generation, however, marks a significant advancement with site-specific conjugation, where cytotoxic drugs are attached at defined positions on the antibody, leading to a more homogeneous ADC with a precise drug-to-antibody ratio (DAR) of either 2 or 4.



While hydrophobic interaction chromatography (HIC) has traditionally been used for DAR determination, this approach may not offer the optimal resolution for third-generation ADCs. These molecules exhibit lower hydrophobicity compared to their first- and second-generation counterparts. In contrast, reversed phase chromatography (RP) provides enhanced resolution for

these less hydrophobic ADCs, as the stronger interactions with the hydrophobic RP stationary phase become advantageous. Moreover, RP facilitates the integration of mass spectrometry detection, offering a streamlined workflow compared to the high salt concentrations typically required in HIC.



This application note demonstrates the accurate DAR determination of a site-specific ADC using a YMC-Triart Bio C4 column, a robust wide-pore hybrid silica-based column designed to deliver reliable, high-resolution separations.

Table 1: Chromatographic conditions.

Column:	YMC-Triart Bio C4 (30 nm, 3 $\mu$ m) 150 x 4.6 mm ID
Part No.:	TB30S03-1546PTH
Eluent:	A) 0.1% TFA in water B) 0.1% TFA in acetonitrile
Gradient:	30%B (0–2.2 min), 30–46%B (2.2–44 min), 46–90%B (44–53 min), 90%B (53–59 min)
Flowrate:	1.0 mL/min
Temperature:	60 °C
Detection:	UV at 280 nm
Injection:	0.7 $\mu$ L
Sample:	Site-specific, 3 <sup>rd</sup> generation ADC (in-house, conc. 20 mg/mL)

Figure 1 demonstrates that the drug conjugation is complete, as no bare antibody can be detected. The majority of the payload has a DAR of 4, and the ADC with a DAR of 2 can be separated from the main peak with high resolution.

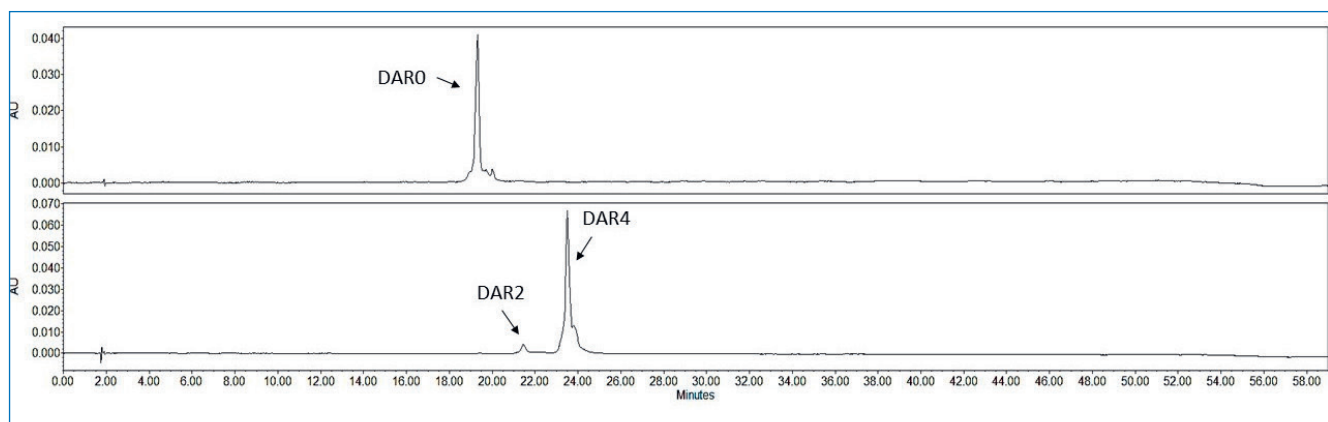


Figure 1: Analysis of a site-specific ADC before (A) and after (B) drug conjugation.

\*Application data by courtesy of KriSan Biotech Co., Ltd.