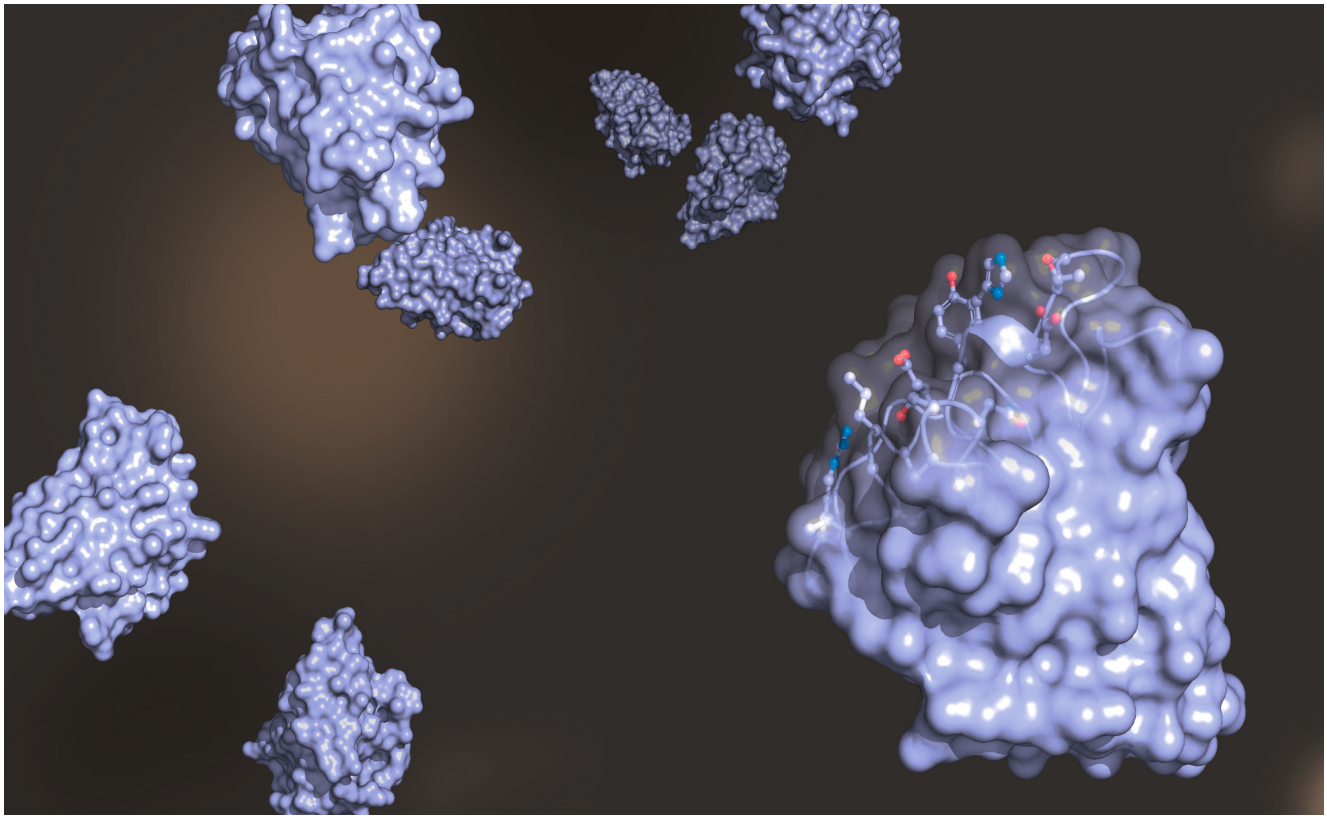
Efficient separation and analysis of antibody fragments and their drug conjugates

Alongside monoclonal antibodies (mAbs) and antibody-drug-conjugates (ADCs) antibody fragments and their drug conjugates find application in therapeutic treatments. These fragments bind selectively to specific antigens, mirroring the function of intact antibodies. Due to their significantly smaller size, antibody fragments require dedicated analytical methods for precise characterisation and quality control.



This Application Note presents a precise method to separate a single-chain variable fragment (scFv) from its conjugated species (FDC). The separation was performed using a SEC-MS method. To achieve optimal resolution and peak shapes, a bioinert column hardware is essential. A metal-free PEEK-lined YMC-Pack Diol-120 column was selected for this analysis, ensuring high performance in the characterisation of the scFv with a molecular weight of approximately 26 kDa.

Table 1: Chromatographic conditions.

Column:	YMC-Pack Diol-120, metal-free PEEK-lined (12 nm, 5 µm) 150 x 4.6 mm ID
Part No.:	DL12S05-1546PTP
Eluent:	75 mM ammonium acetate/ 4.5% 2-propanol (UV)
	75 mM ammonium acetate/ 10% 2-propanol (MS)
Flow rate:	0.3 mL/min
Temperature:	ambient
Injection:	1 µL
Sample:	0.5 mg/mL scFv
	0.5 mg/mL FDC
	0.5 mg/mlL scFv+ FDC mix
Detection:	UV at 280 nm
	ESI-MS positive mode

Figure 1 illustrates the separation of the scFv and FDC, injected individually and as a mixture, each at a concentration of 0.5 mg/mL, without isopropanol in the mobile phase. The target FDC-to-scFv ratio, based on peak area, is approximately 0.8. Table 2 shows that this ratio is not achieved under these conditions, either for individual injections or for the mixture.

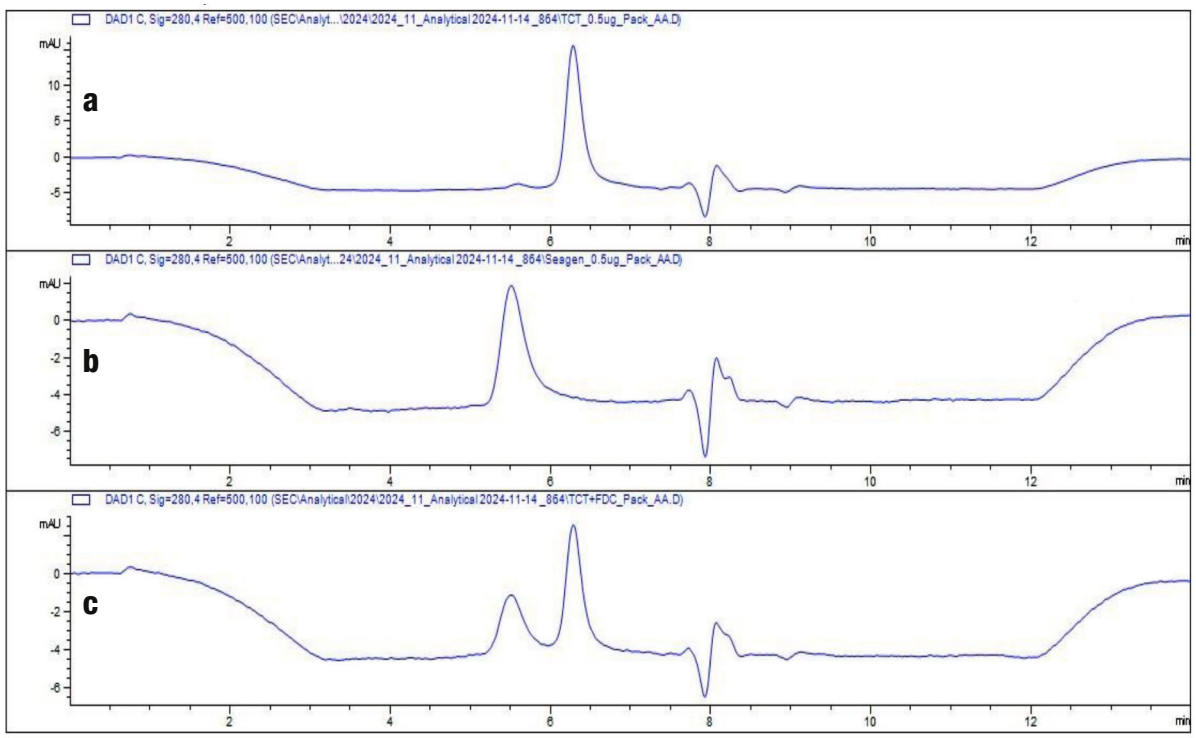


Figure 1: Chromatograms from the analysis of the unconjugated protein scFv (a), the conjugated protein FDC (b) and a mixture of both (c) using 75 mM ammonium acetate as eluent.

Table 2: Peak areas and calculated ratios of the FDC to the scFv using 75 mM ammonium acetate as eluent.

	Time	Area	FDC-to-scFv ratio
scFv	6.28	315.8	
FDC	5.51	149.4	0.47
scFv + FDC	6.28	95.4	
scFv + FDC	5.50	58.2	0.61

The addition of 4.5% isopropanol to the mobile phase increases the peak area of the hydrophobic FDC, allowing the ratio of 0.8 to be reached in both cases (Figure 2, Table 3). Additionally, isopropanol improves peak symmetry, further enhancing separation performance.

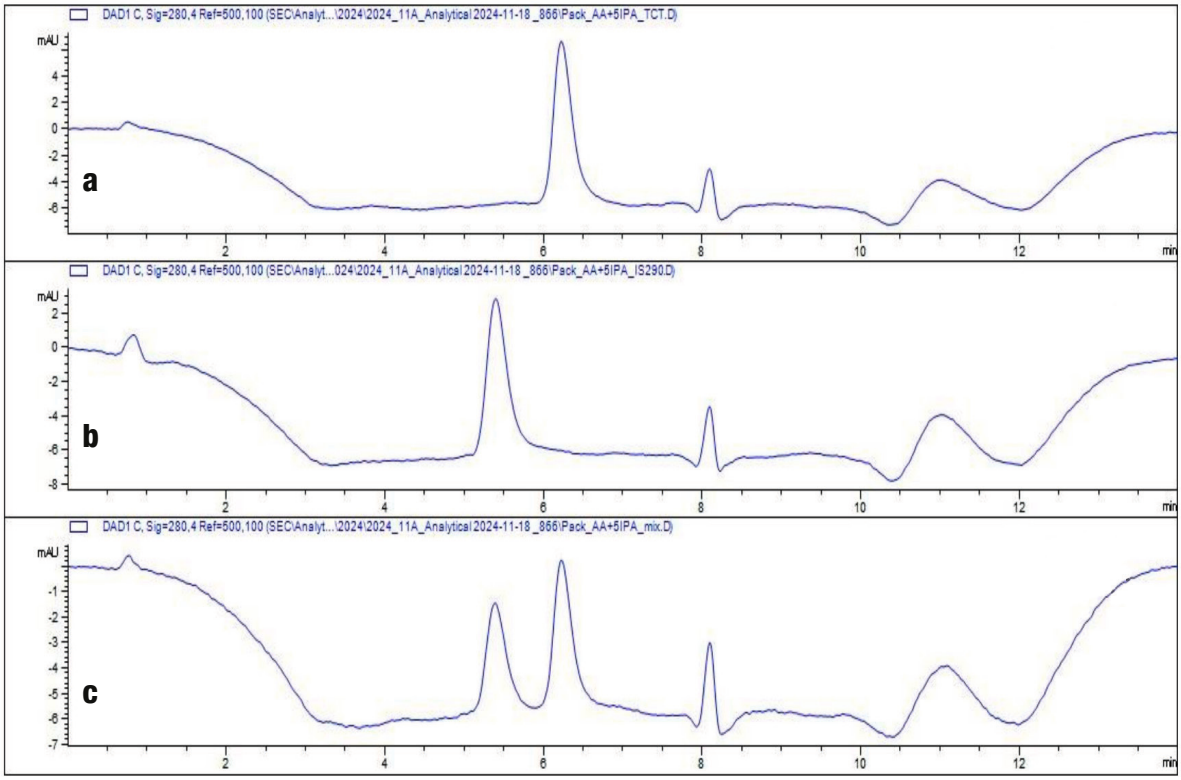


Figure 2: Chromatograms from the analysis of the unconjugated protein scFv (a), the conjugated protein FDC (b) and a mixture of both (c) using 75 mM ammonium acetate/ 4.5% 2-propanol as eluent.

Table 3: Peak areas and calculated ratios of the FDC to the scFv using 75 mM ammonium acetate/ 4.5% 2-propanol as eluent.

	Time	Area	FDC-to-scFv ratio
scFv	6.23	220.3	
FDC	5.39	181.5	0.82
scFv + FDC	6.22	97.0	
scFv + FDC	5.38	74.2	0.76

The deconvoluted mass spectra of the scFv and FDC shown in Figure 3 and 4 confirm the separation of both compounds. That makes this method an ideal tool to monitor the conjugation of the scFv.

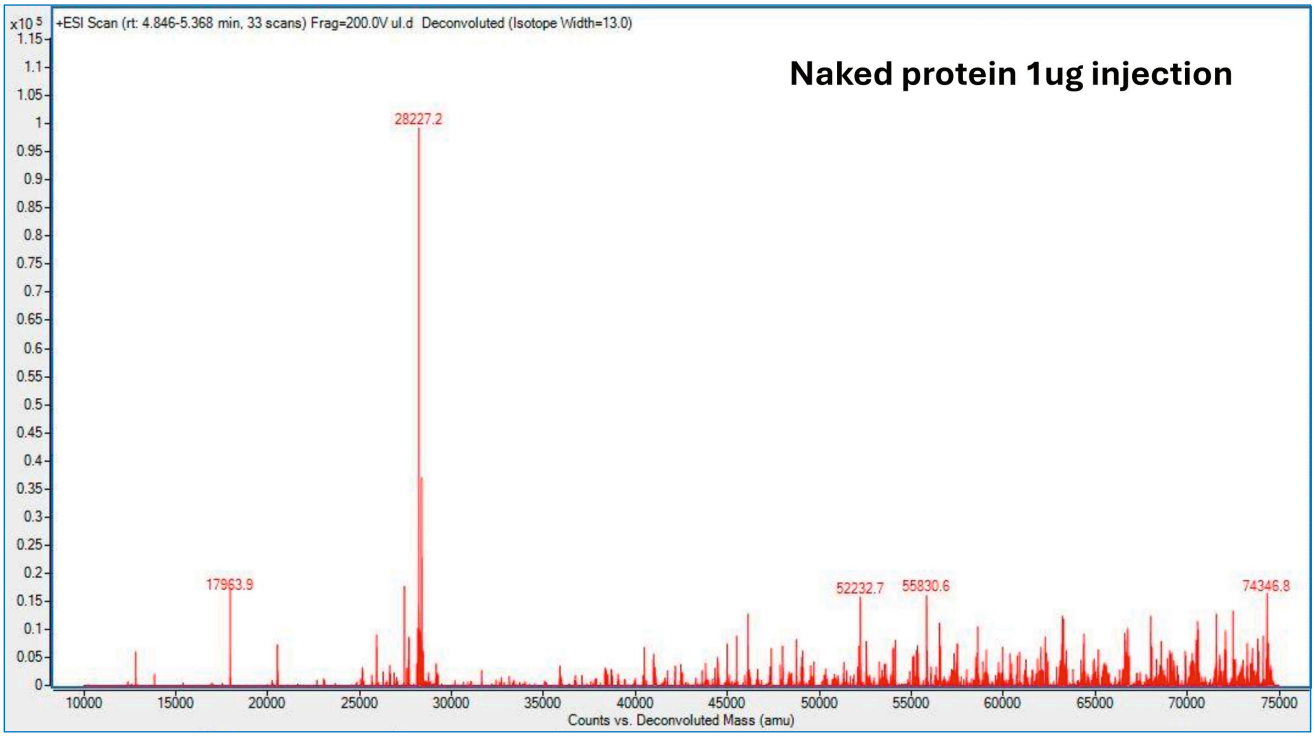


Figure 3: Deconvoluted mass spectrum of the scFv at 1 µg load.

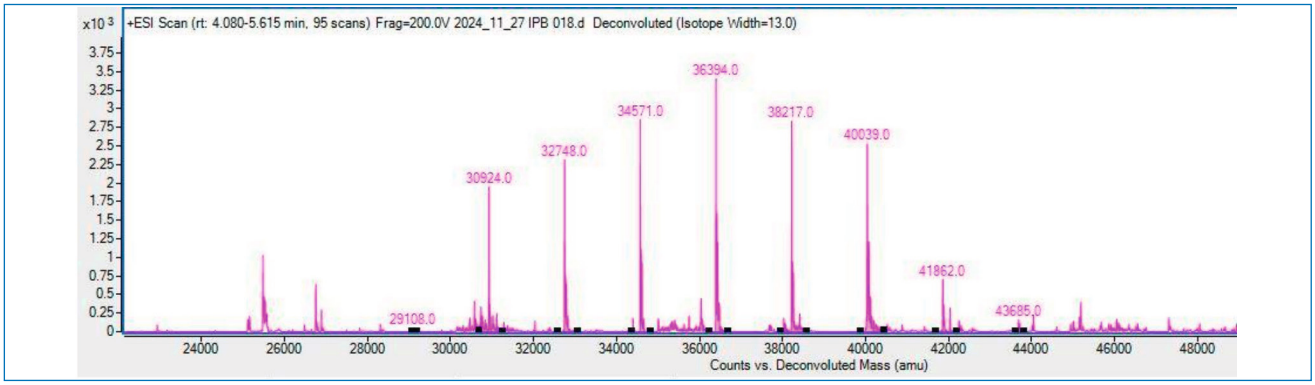


Figure 4: Deconvoluted mass spectrum of the FDC at 1 µg load.

**Application data by courtesy of Laura Bouché and Anja Pomowski, ANTIKOR, Stevenage, United Kingdom*