Which LC-mode is more suitable for different ADC-types? RP or HIC?

The chromatographic analysis of antibody-drug-conjugates (ADCs), especially the determination of the drug-to-antibody ratio (DAR), is essential to guarantee the efficacy and minimise the toxicity at the same time. There are different methods to produce ADCs and therefore different conjugated ADCs with altered potency characteristics are possible.
Currently, hydrophobic interaction chromatography (HIC) is the standard mode for ADC analysis. However, reversed phase chromatography (RP) could take in a greater role in the future, as shown by way of this example.
Both modes use hydrophobic interactions for the separation of the analytes. In HIC mode salt gradients are used for the separation. High salt concentrations strengthen the interactions whereas low concentrations lead to elution. The more hydrophobic the biomolecule is the less salt is necessary to encourage the binding.
In RP mode biomolecules are eluted from the column according to their hydrophobicity by increasing the organic ratio. As hydrophobic interactions in RP mode are stronger, nonpolar organic solvents such as acetonitrile are required to elute the biomolecules.

ADC types

Antibody-drug conjugates (ADCs) are therapeutic agents with a high potential for cancer treatment due to their more targeted mode of action. An ADC is based on a recombinant monoclonal antibody (MAb) with cytotoxic drugs (also called “payload”) covalently bonded to it.

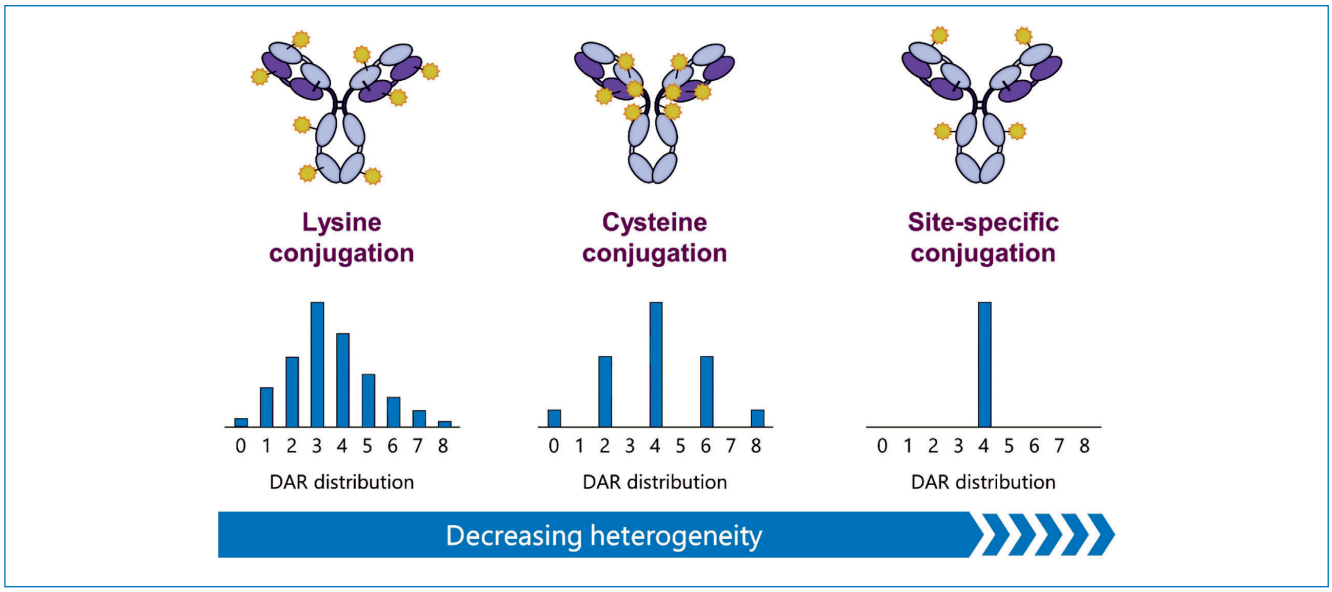


Figure 1: Schematic representation of the first (lysine conjugation), second (hinge cysteine conjugation) and third (site-specific conjugation) generations of ADC products. The theoretical DAR distribution of the three ADC types was also highlighted. (1)

There are three types of ADCs that are currently available on the market.

1. Lysine conjugation (1st generation):

- Cytotoxic drugs are randomly attached to amine residues of lysine side chains
- More than 60 surface exposed lysine residues are available
- Highly heterogeneous
- DAR between 0 and 8
- Characterisation is challenging
  ➪ **Only HIC** is possible
- Examples: trastuzumab-emtansine (Kadcyla®), gemtuzumab-ozogamicin (Mylotarg®)

2. Cysteine conjugation (2nd generation):

- Uses cysteine residues of reducible inter-chain disulphide bridges (between two heavy chains or heavy and light chain)
- More homogeneous population
- DAR between 0 and 8
- Average DAR can be controlled – equal to about 4 (e.g. brentuximab-vedotin)
  ➪ **HIC** gold standard for drug distribution, intact antibody and average DAR
- Examples: brentuximab-vedotin (Adcetris®), enfortumab-vedotin (Padcev®)

3. Site-specific conjugation (3rd generation):

- Payload attached to defined positions suitable for drug conjugation
- Improving therapeutic index
  · Higher potency
  · Lower toxicity
- More than 40 site-specific drug conjugate technologies developed
- Additional cysteine residues engineered into different sites of the MAb
- Near-uniform stoichiometry of cytotoxic molecules attached
- No disruption of inter-chain disulphide bonds
- Average DAR near 2 or 4
- In practice more heterogeneous due to different payload accessibility and possible artefacts during synthesis
  ➪ **HIC** still used routinely
  ➪ **RP** can provide better resolution
- Examples: substances are currently in R&D, clinical phases

Advantages and disadvantages of HIC and RP in ADC analysis

HIC:

HIC resins are most commonly hydrophilic polymer particles functionalised with hydrophobic groups. As a result HIC is especially suitable for separating hydrophobic biomolecules.

- ✔ **Non-denaturating conditions**
  - ➪ MAbs can be analysed in their native structure
- ✗ **High salt gradients used**
  - ➪ No direct coupling to MS possible (2D-LC required)
- ✗ **Limited kinetic efficiency**
  - ➪ Separation of other than native linked ADC species is challenging
- ✗ **Insufficient resolution to separate positional isomers**

RP:

The packing materials for RP analyses usually are based on porous, hydrophobic silica or hybrid silica which are also functionalised with hydrophobic groups. Strong hydrophobic biomolecules interact too strongly with the stationary phase with the result that no separation is possible. Therefore, RP is generally more suitable for less hydrophobic biomolecules.

- ✔ **No need for sample preparation**
- ✔ **Direct coupling to MS possible**
- ✔ **High resolution**
- ✗ **Mostly denaturating conditions**

For both modes YMC provide the ideal solution: BioPro HIC HT for fast and reproducible HIC analyses and YMC-Triart Bio C4 for the analyses using RP.

	BioPro HIC HT	YMC-Triart Bio C4
Base particle	polymethacrylate	Fully porous organic/ inorganic hybrid silica
Modification	C4	C4
Particle size [µm]	2.3	1.9, 3, 5
Pore size [Å]	Non-porous	300
Endcapping	–	yes
pH limit	2–12	1–10
Temperature limit [°C]	10–60	pH < 7: 90, pH > 7: 50

Example of a site-specific ADC analysed with HIC and RP

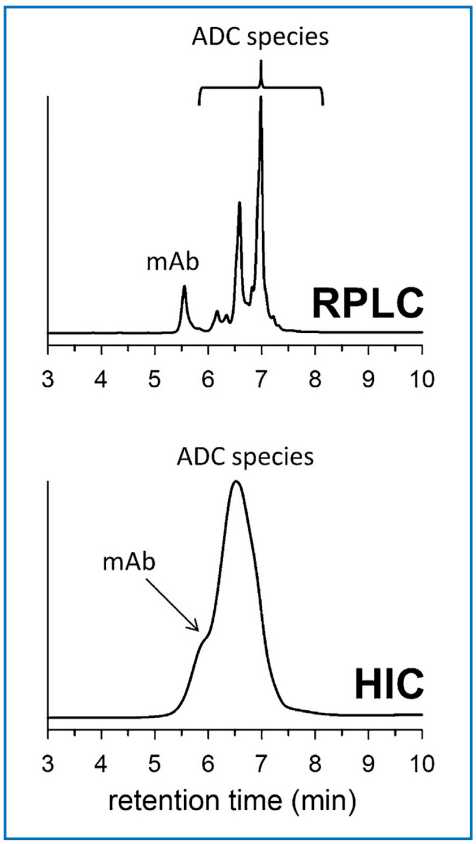
This example demonstrates the benefits of RP compared to HIC when analysing a heterogeneous population of a site-specific cysteine-conjugated ADC.
Furthermore, the nonconjugated MAb needs to be separated from the ADC as well as DAR species, so that the average DAR and distribution can be determined.

Sample:

IgG1 MAb with two identical light chains and heavy chains linked by disulphide bonds

- Heavy chains consist of an N-terminal variable domain (VH) and three constant domains (CH1, CH2, CH3)
- With engineered cysteines (E157C) in CH1 and (S380C) in CH3 allowing site-specific conjugation
- Linker and payload connected via disulphide bridge

Results:



In Fig. 2 the chromatographic results for the RP and HIC separation of a site-specific cysteine-linked ADC sample spiked with 10 % nonconjugated MAb are shown.

RP:

- Nonconjugated MAb is base line separated from early eluting DAR species
- DAR2, DAR3, DAR4 can be discriminated
  · Their variants are partly resolved
- Average DAR and DAR distribution can be determined
  · 7.4 % DAR2, 37 % DAR3, 55.6 % DAR4
- Measurement of DAR distribution is generally not possible for conventional cysteine-linked ADCs as they are too hydrophobic

HIC:

- All ADC species coelute
- Unconjugated MAb can only be partly resolved

Figure 2: RP and HIC separations of site-specific cysteine-linked ADC sample spiked with 10% nonconjugated MAb. (1)

Conclusions drawn from these results:

- Currently HIC is the standard technique for strong hydrophobic ADCs (cysteine conjugation)
- RP shows much potential for site-specific MAbs
  · Higher resolution can be achieved than when using HIC
  · Due to direct MS coupling ADC species can be analysed
  · Nonconjugated MAb and ADC can be separated
  ➪ DAR distribution can be determined
  · Modern materials can achieve high resolution even under mild conditions
  ➪ Degradation avoided

Chromatographic conditions:

100 mm column length and 10 min gradient for both modes

RP:

Mobile phase:	(A) water + TFA/FA (0.05%/0.05%)
	(B) acetonitrile + TFA/FA (0.05%/0.05%)
Gradient:	30–46 % B in 10 min
Temperature:	60 °C
Detection:	fluorescence ex. 280 nm, em. 350 nm

HIC:

Mobile phase:	(A) 1.5 M ammonium sulphate containing 0.1 M phosphate buffer
	(B) 0.1 M phosphate buffer
Gradient:	2–75 % B in 10 min
Temperature:	ambient
Detection:	fluorescence ex. 280 nm, em. 350 nm

Refernce:

(1) V. D'Atri, R. Pell, A. Clarke, D. Guillarme, S. Fekete, Is hydrophobic interaction chromatography the most suitable technique to characterize site-specific antibody-drug conjugates?, J. Chrom. A (2018)