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Intact Analysis of Biopharmaceuticals by Hydrophobic Interaction/Reversed Phase 2D-LC/MS System

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Introduction

Hydrophobic Interaction chromatography (HIC) is a popular LC technique widely used in downstream process purification but recently gained interest in analytical scale analysis of mAbs and ADCs. HIC technique can be applied to separate mAb variants such as oxidation and isomerization species, which are often different to isolate from other orthogonal chromatography methods. It also a popular tool in determining drug-to-antibody ratio (DAR) value which is considered quality assessment of an ADC product. Such investigations often benefit from mass measurement using Mass Spectrum (MS) detection, but the high salt conditions used for HIC separations make it incompatible to couple online MS.

In this presentation, we present 2D-LC approach to overcome this obstacle, which affords multiple heart-cutting (MHC) and subsequent desalting/separation using reversed-phase chromatography on-line with TOF MS were explored.

Experimental

Sample Preparation

For Biosimilar mAb1 and ADC, samples were analyzed directly. mAb2 was subject to forced oxidation using 1% T-Butyl Hydro Peroxide (TBHP) for 72 hours at 37 °C.

Instrumentation

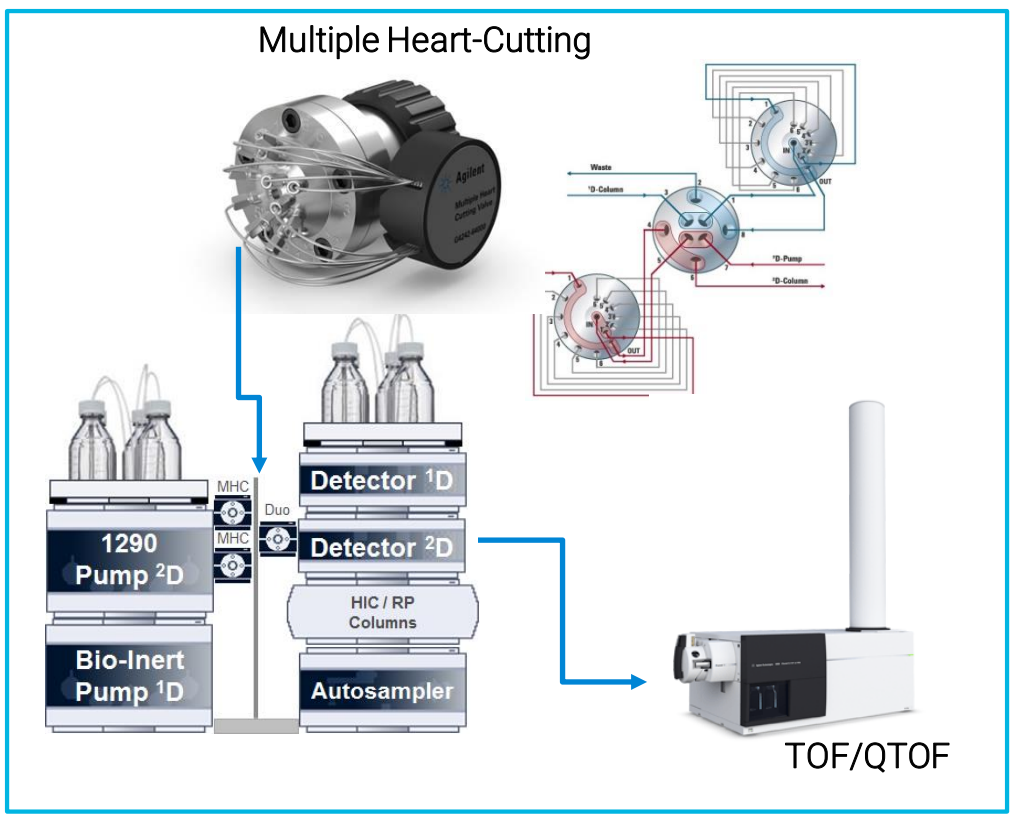


Figure 1. Samples were analyzed on the Agilent 1290 Infinity 2D-LC Solution using an Agilent 1260 Infinity II BioInert LC system on ^{1}D and 1290 Infinity II LC system on ^{2}D coupled with an Agilent 6224 TOF LC/MS system with dual-nebulizer ESI source.

Experimental

HIC/RP 2DLC/MS Separation of mAbs and ADC

LC Conditions – HIC ^{1}Dimension parameters	
Column	Agilent AdvanceBio HIC, 4.6 x 100 mm 3.5 µm, p/n 685975-908
Column Temp	25 °C
DAD	280 nm
Injection Vol.	1 -10 µL
Auto sampler Temp	4 °C
Mobile Phase	A = 50 mM Sodium Phosphate pH 7.0 B = 2 M Ammonium Tartrate in A pH 7.0 C = Isopropanol
^{1}D Flow Rate	0.4 mL/min

Gradient Program	Project 1			Project 2			
	Time	% A	%B	Time	%A	%B	%C
	2	25	75	0	25	75	0
	17	100	0	15	75	0	25
	20	100	0	20	75	0	25
	22	25	75	21	25	75	0
	25	25	75	25	25	75	0

LC Conditions – RP ^{2}Dimension Parameters	
Column	Agilent AdvanceBio RP-mAb C4, 2.1 x 50 mm, p/n 799775-904
Column Temp	80 °C
^{2}D Flow Rate	0.2 mL/min
2D-LC mode	Multiple heart-cutting
^{2}D Gradient stop	5 minutes
^{2}D cycle time	7 minutes
Mobile Phase	A = 0.5% Acetic acid, 0.05% TFA in water B = 0.5% Acetic acid, 0.05% TFA in 80:10:10 acetonitrile/1-propanol/water
Valve and loop configuration	2- position / 4-port-duo 2x6 loops (concurrent); Loop size: 40 µL

Gradient Program	Time	% B
	2	28
	4	42
	4.6	50

Data Analysis

Data was analyzed using Agilent Open LAB CDS ChemStation Edition Rev. C.01.07 and Agilent MassHunter workstation software, version B.07.01.

Project 1: HIC/RP-2DLC/MS Analysis of Biosimilar mAb1 and Oxidized mAb2

In HIC separations, proteins interact with a weakly hydrophobic stationary phase in the presence of a high initial concentration of lyotropic or cosmotropic salts. The concentration of this salt is lowered as the gradient proceeds, resulting in elution of the protein from the stationary phase.

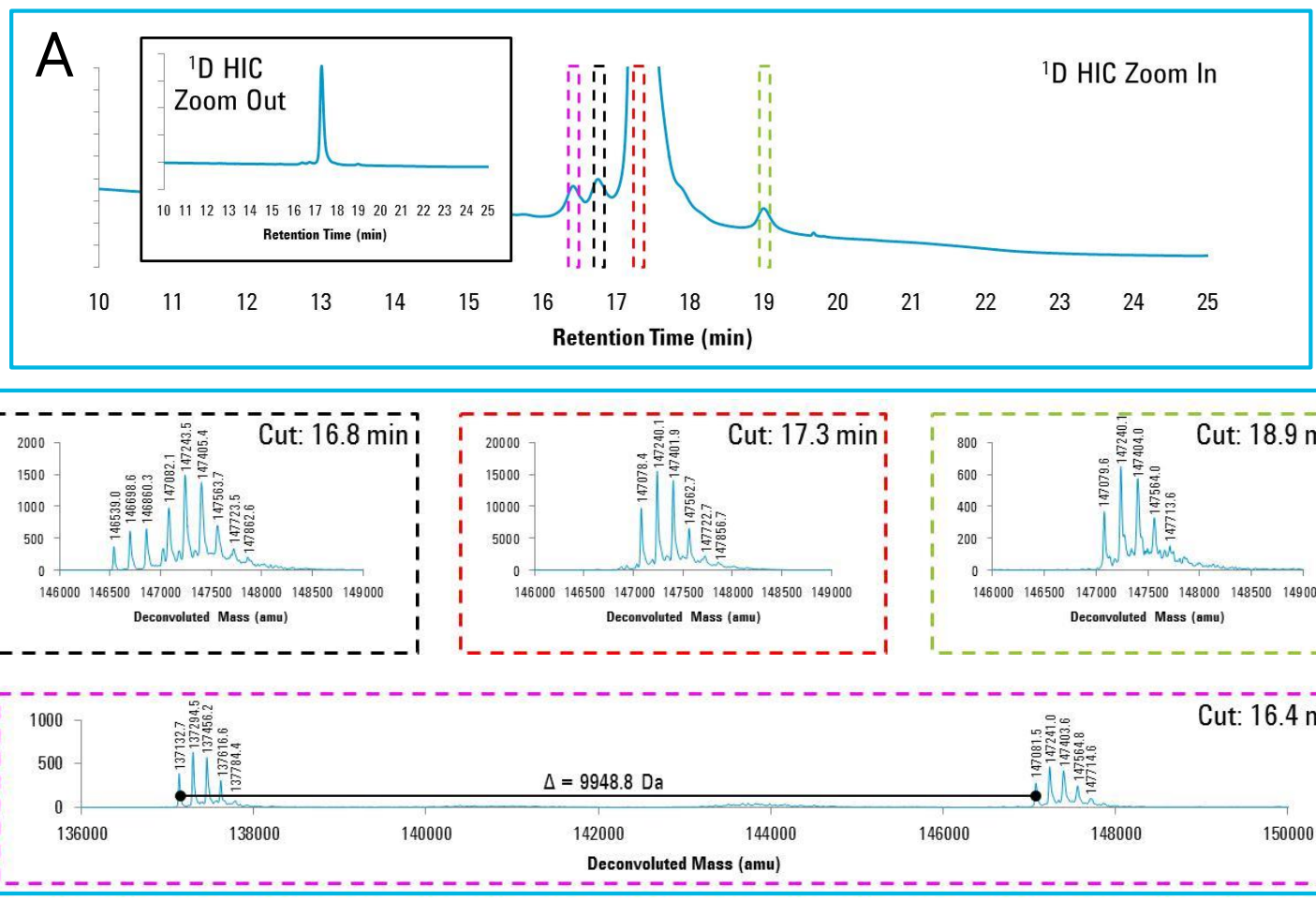


Figure 2. Analysis of Biosimilar mAb1. (A) HIC chromatogram of Biosimilar mAb1 revealing multiple satellite peaks (see zoom in) as well as one major peak (see zoom out). (B) Heart-cuts from the first dimension at 16.4, 16.8, 17.3, and 18.9 minutes were stored, then separated on the second dimension followed by MS detection. Various impurities were detected that ranged in mass difference from the main peak. From the data shown above, it appears that the earliest eluting peaks (16.4 min) represent truncated variants of the mAb, while the latest eluting peak may represent an isomer having significant differences in surface hydrophobicity from the main mAb peak.

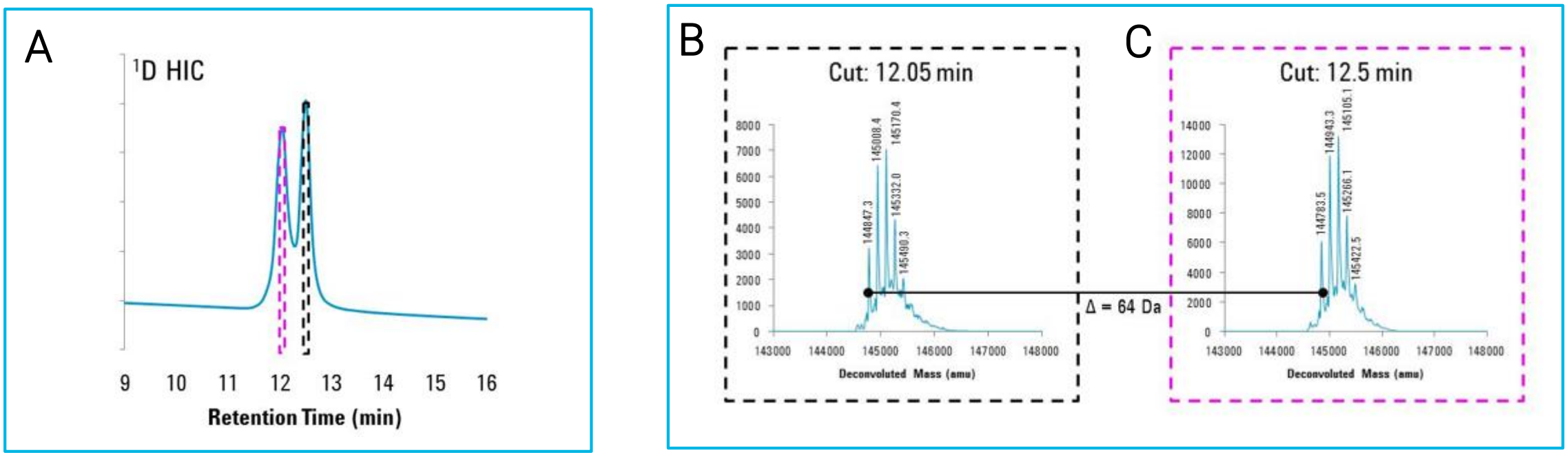
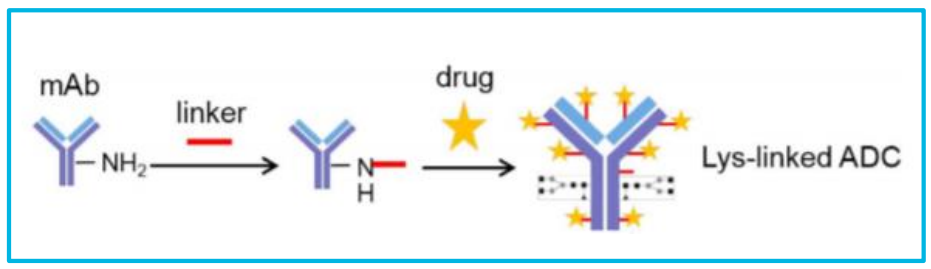


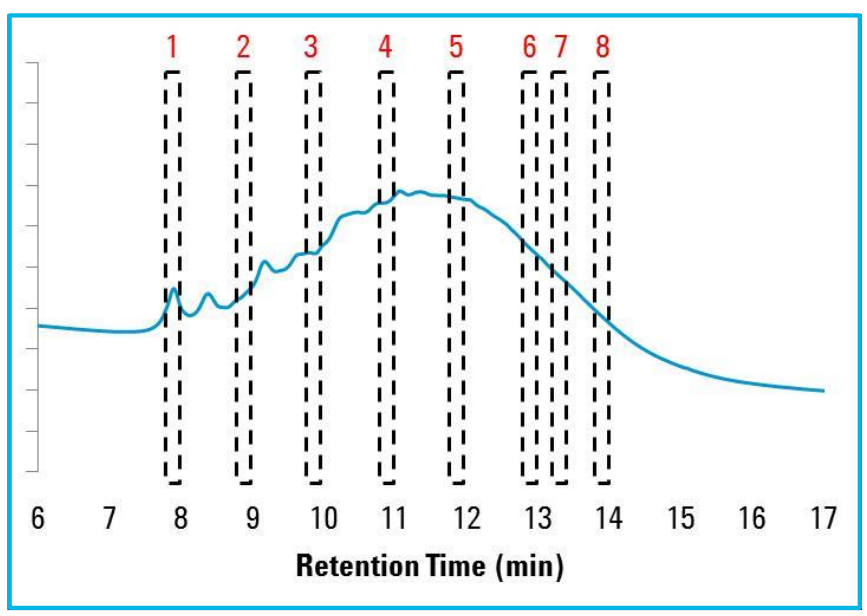
Figure 3. Analysis of Forced Oxidized mAb2. (A) HIC chromatogram of mAb2 treated with 1 % TBHP. TBHP treatment resulted in a new peak at RT 12.05 minutes. (B) Mass measurement of the peak at 12.05 minutes. (C) Mass measurement of the peak at 12.5 minutes. The mass of the oxidized mAb was 64 Da higher than the untreated mAb, indicating four potential sites of oxidation. These oxidation events are likely to occur at the four methionine residues present in the mAb, which are susceptible to this modification.

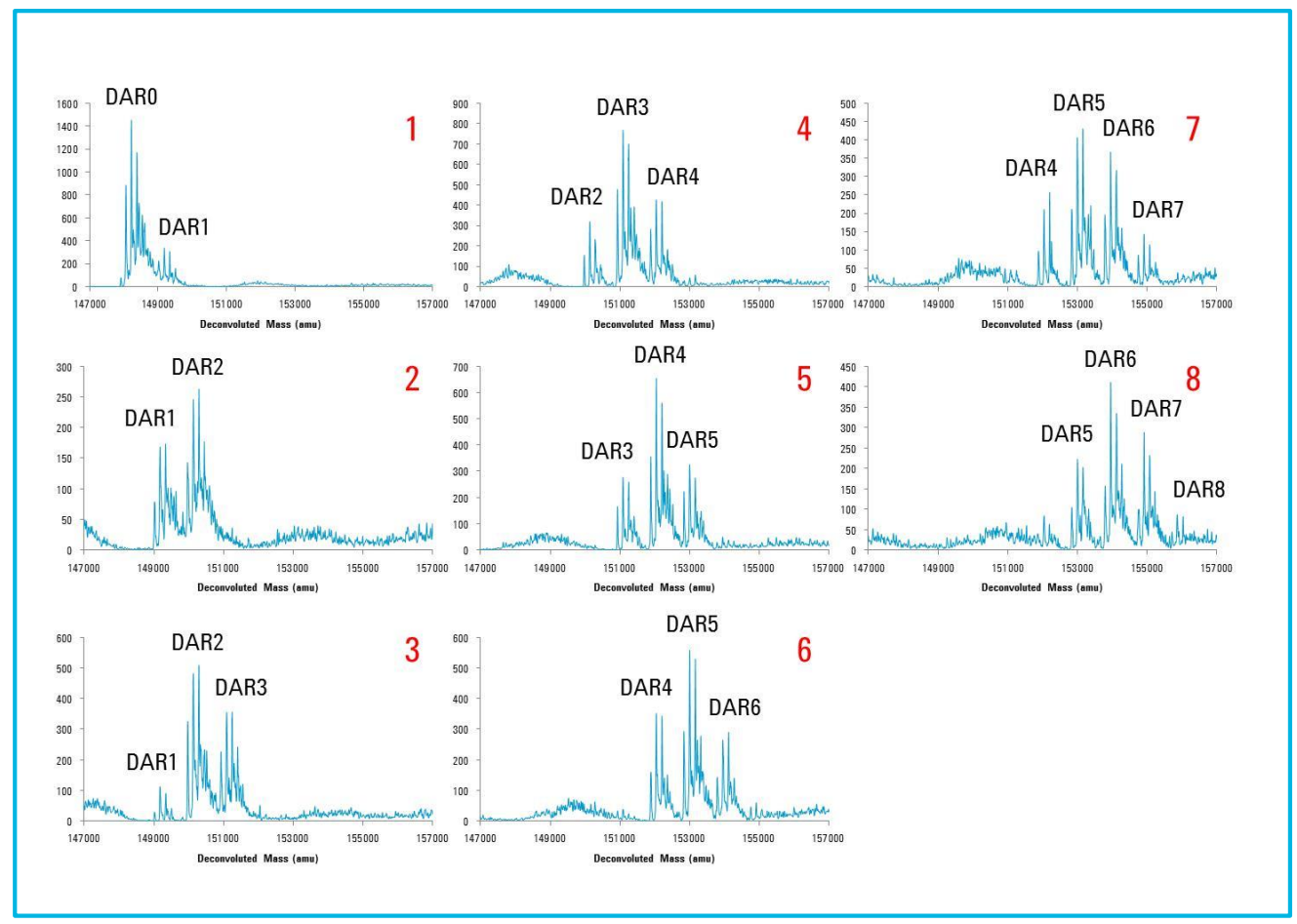
Results and Discussion

Project 2: HIC/RP-2DLC/MS Analysis of Lys-linked ADC

Figure 4. A Lysine-linked ADC was analyzed using the HIC/RP 2D-LC/MS system. Lys-linked ADC sample is illustrated as cartoon below. No sample deglycosylation was performed prior to analysis. The resulting HIC chromatogram consisted of a very poorly resolved group of peaks eluting over the course of ~7 minutes, reflective of the multiple positional isomers present in the preparation. Eight heart-cuts at RTs 7.9, 8.9, 9.9, 10.9, 11.9, 12.9, 13.4, and 13.9 min were selected and sent to the [2]Dimension Reverse phase separation to TOF MS.







The MS spectra corresponding to the heart-cuts are shown to right. mAbs with DAR ranging from 0-8 were observed. The deconvoluted mass spectra revealed that RT increases as a function of DAR, as expected. It is also evident, based on the fact that certain DAR species are present in multiple heart-cuts, that there is some selectivity based on the positional isomers in the mixture. Species containing between 0 (unconjugated mAb) and a maximum of 8 small molecule drugs were detected. The spectra also reflect the glycoform distribution of the mAb in addition to a subpopulation of molecules modified by linker without payload.

Summary

- AdvanceBio HIC column proves to be an incredibly useful tool for characterizing mAbs, even when modified components are present as minor species.
- Multiple heart-cutting 2D-LC permits thorough investigation of high salt-driven [1]D bio-column separations.
- Easy to implement mass measurement of HIC separations of mAbs, oxidized mAbs, and ADCs.

References

Staples, E.; Gregory,. Analysis of Monoclonal Antibodies using Multiple Heart-cutting Hydrophobic Interaction/Reversed Phase 2D-LC/MS. Agilent Technologies, Application Note (5991-6376EN). 2016.

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