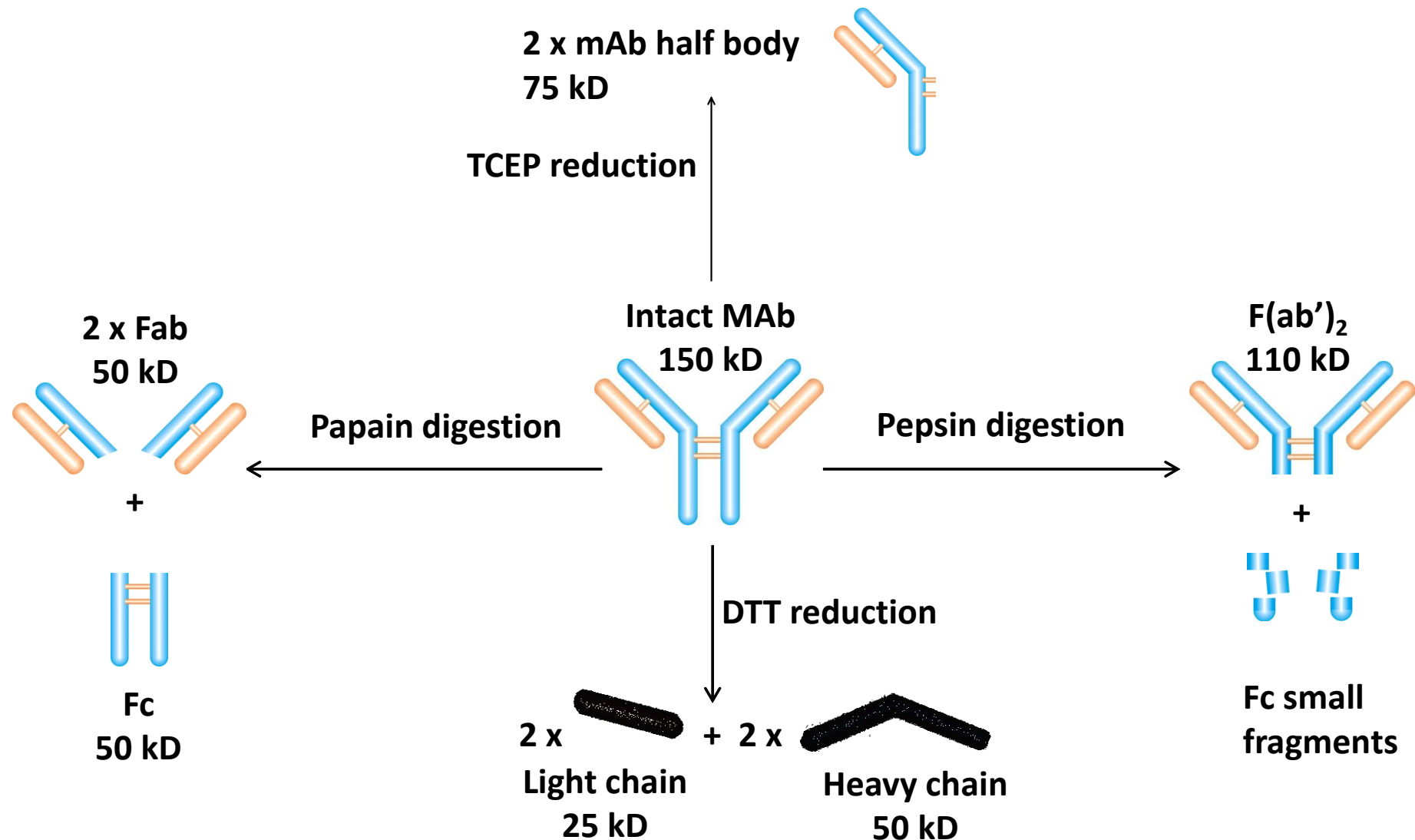


**Zenix-300 (3 μm , 300 Å)
for mAb,ADC fragments separation
with direct mass spec detection**



MAb Fragments

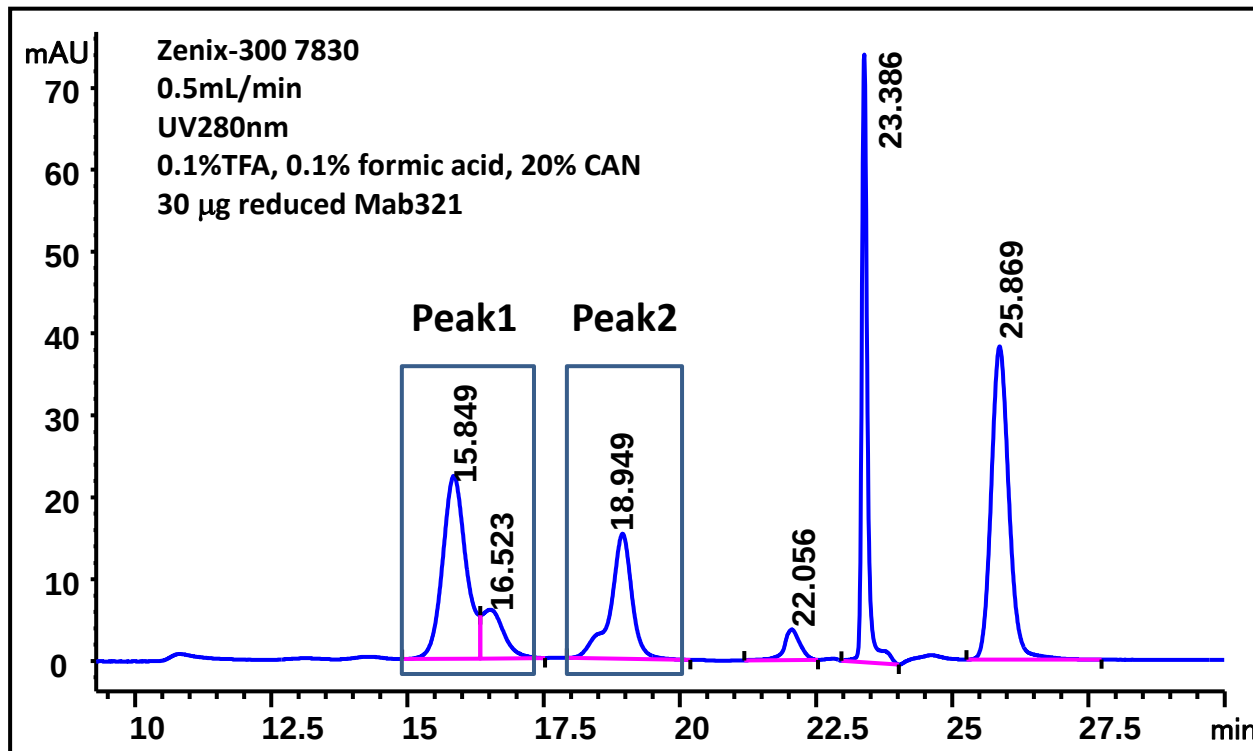


Reduced MAb
Heavy and light chain
Low pH, organic mobile phase

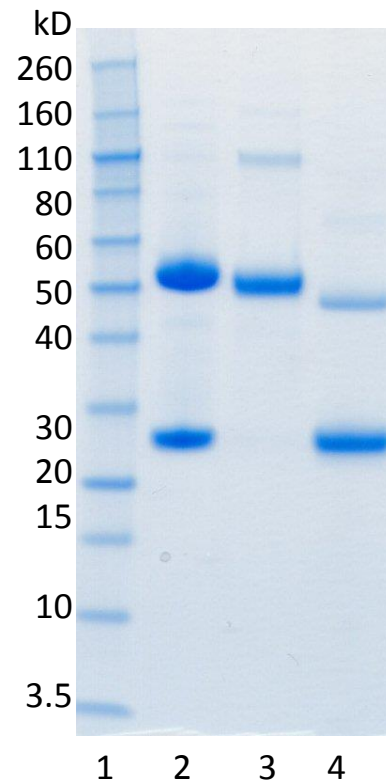


Heavy and light chain of Mab321 on Zenix-300

1. Peak 1 and Peak 2 were collected and dried with speed vac.
2. Re-dissolve in SDS-PAGE gel sample buffer and run gel.



Heavy chain 50 kD, light chain 25 kD

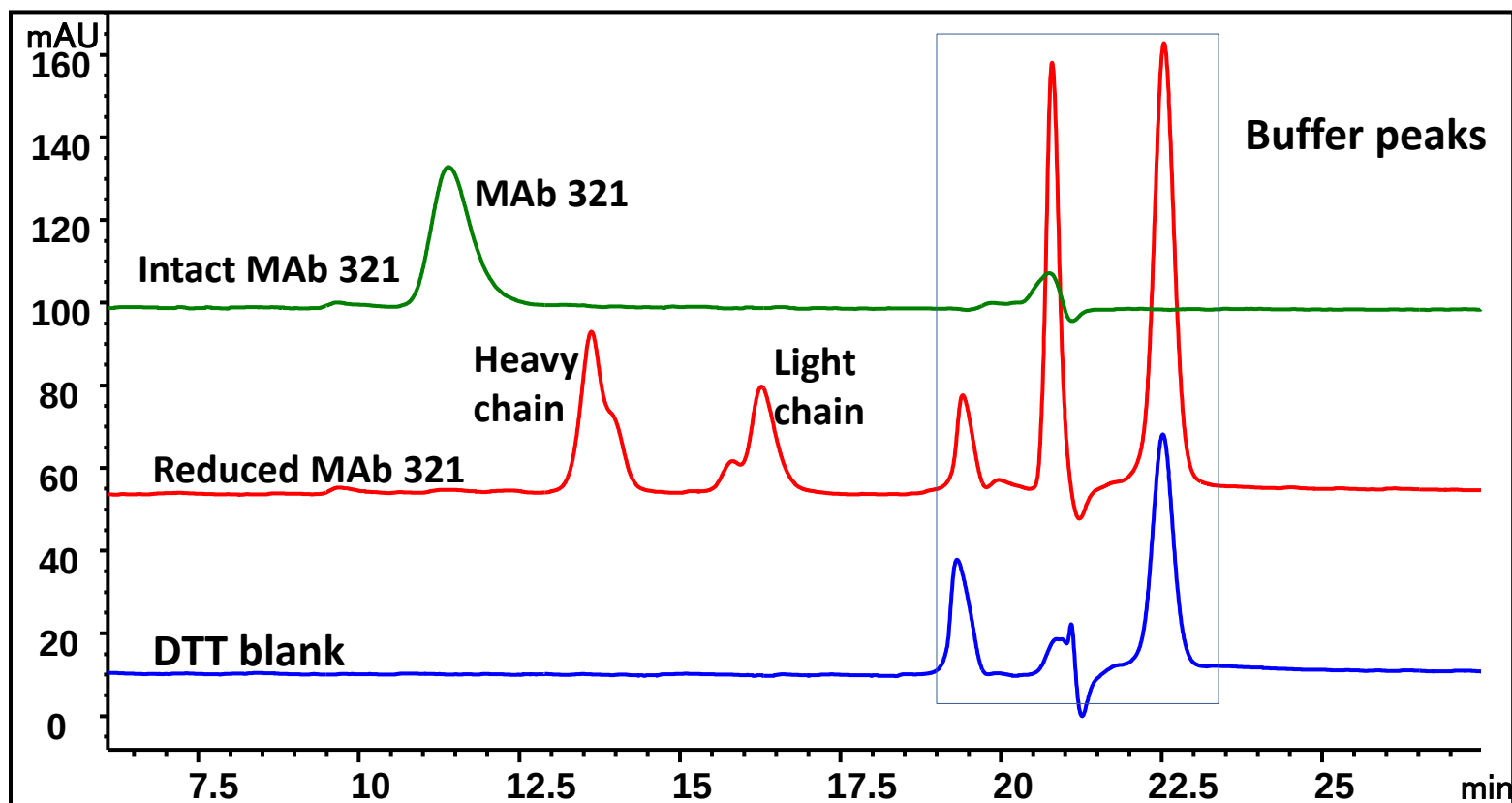


1. Protein marker
2. Reduced Mab321
3. Peak 1 heavy chain
4. Peak 2 light chain

Reduced MAb on Zenix-300 4630

Column: Zenix-300, 4630, Mobile phase: 0.1% TFA, 0.1% formic acid, 20% ACN

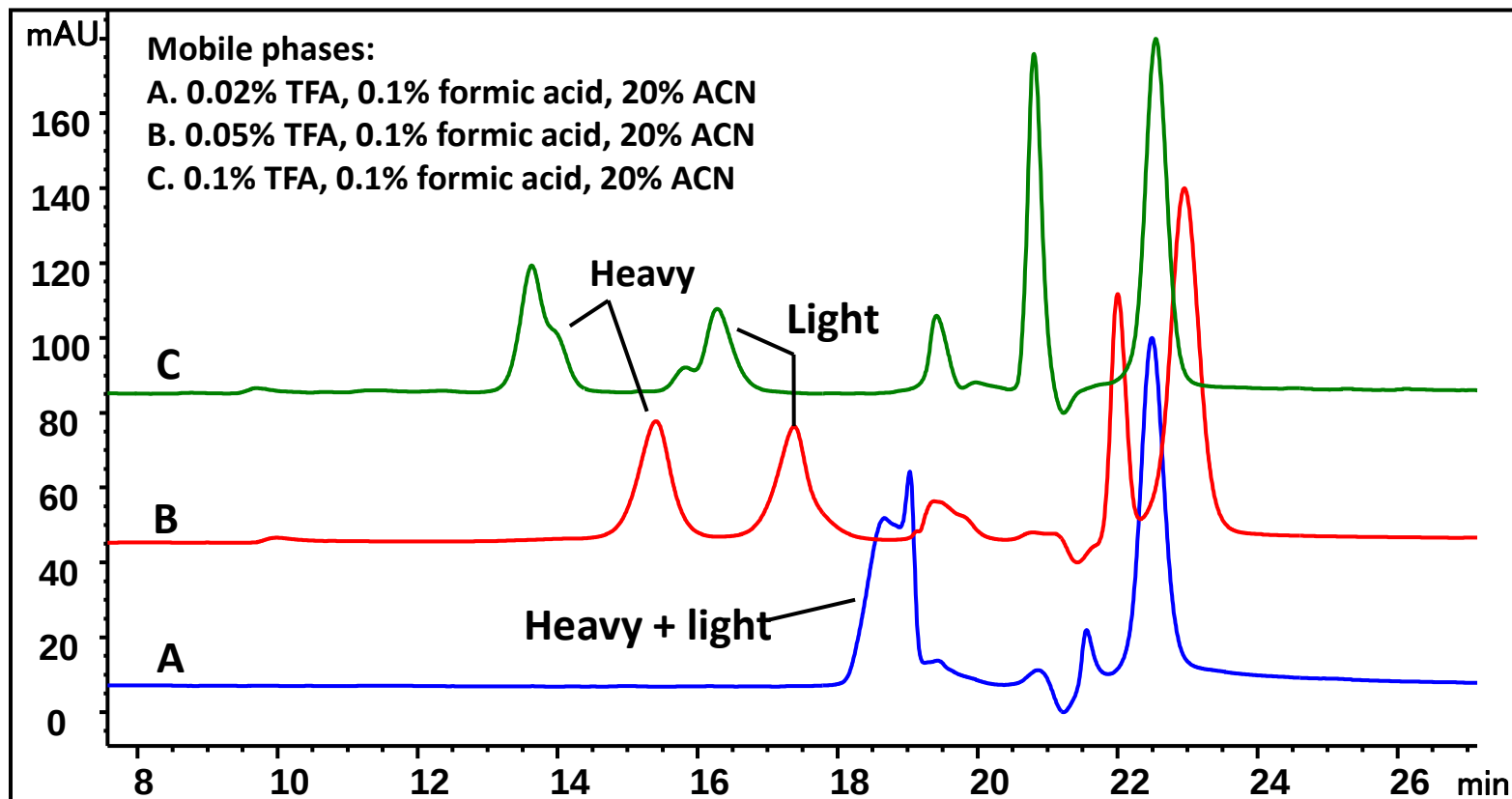
Flow rate: 0.2 mL/min, injection: 5 μ g intact MAb 321, 20 μ g reduced MAb 321 (65°C incubation with 20 mM DTT for 15 minutes)



Different TFA concentration effect on reduced MAb separation on Zenix-300 4630

Column: Zenix-300, 4630, mobile phase: as indicated

Flow rate: 0.2 mL/min, injection: 20 µg reduced MAb 321

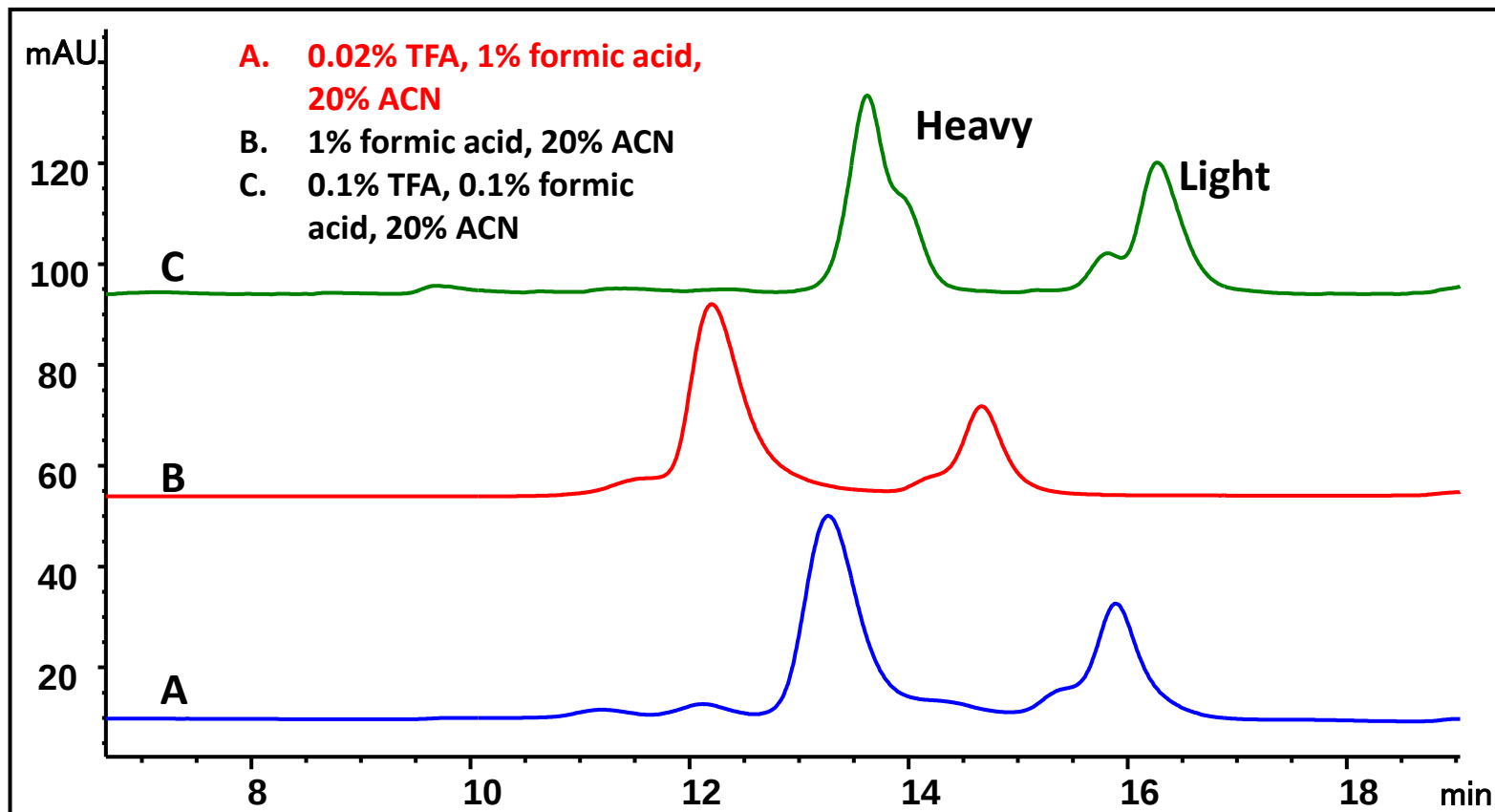


At 0.05% TFA concentration, heavy and light chains can be separated on Zenix-300 4.6 x 300 mm.

Reduced MAb-mobile phases

Column: Zenix-300, 4630, mobile phase: as indicated

Flow rate: 0.2 mL/min, injection: 20 μ g reduced MAb 321

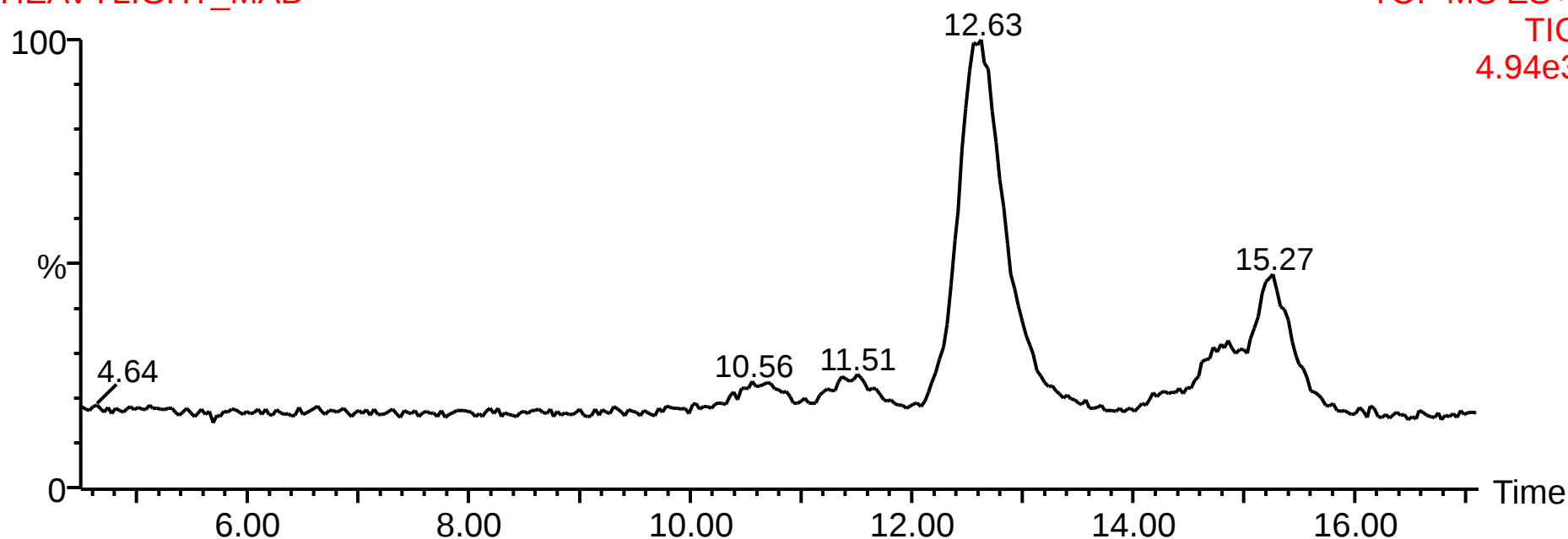


SEC-MS (heavy light chains), TIC

Waters Q-ToF Ultima. The scan range is from 350 to 3000 m/z. The instrument settings are: source temperature = 80°C, desolvation temperature = 150°C, capillary voltage = 4.44 kV

HEAVYLIGHT_MAB

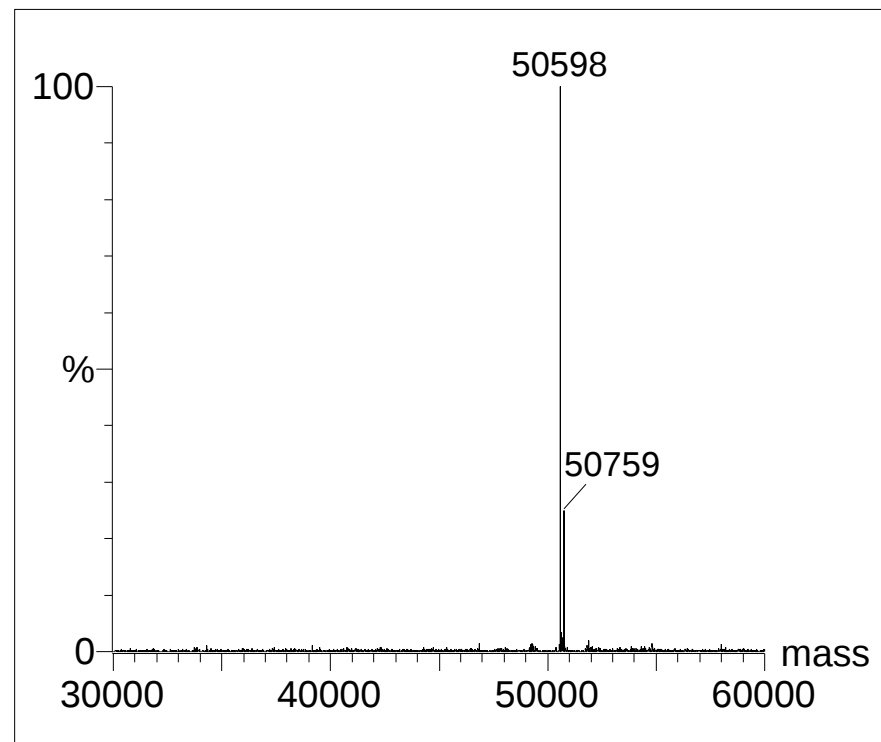
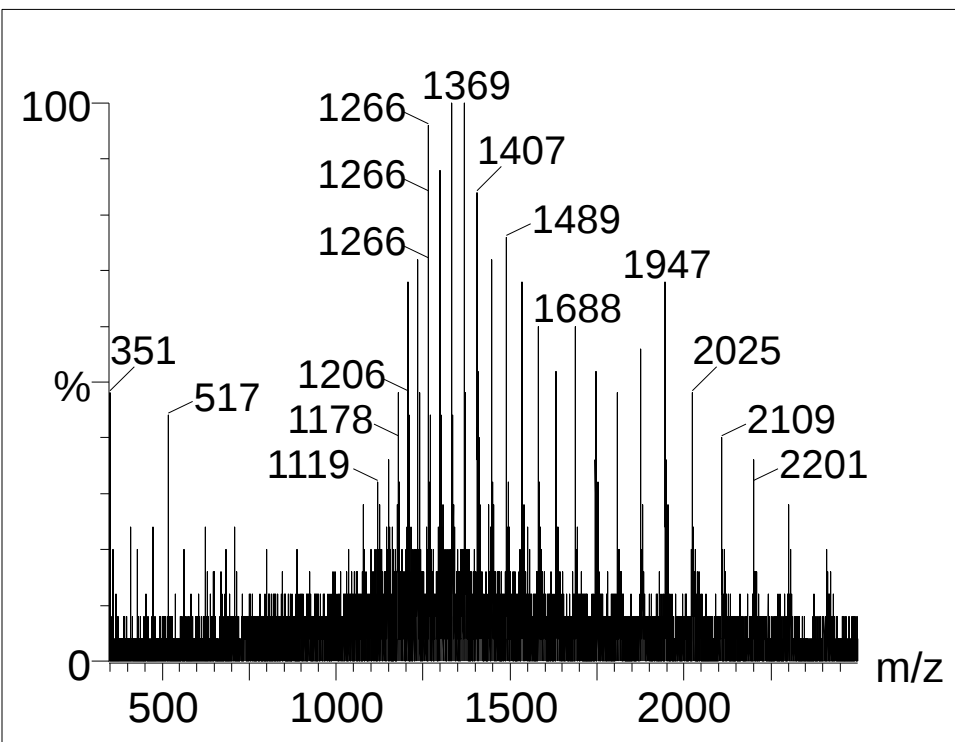
TOF MS ES+
TIC
4.94e3



Column: Zenix-300, 4630, mobile phase: 0.02% TFA, 1% formic acid and 20% ACN
Flow rate: 0.2 mL/min, injection: 10 µg reduced MAb 321

Heavy Chain

HEAVY_MAB 373 (12.626)



162 Galactose difference



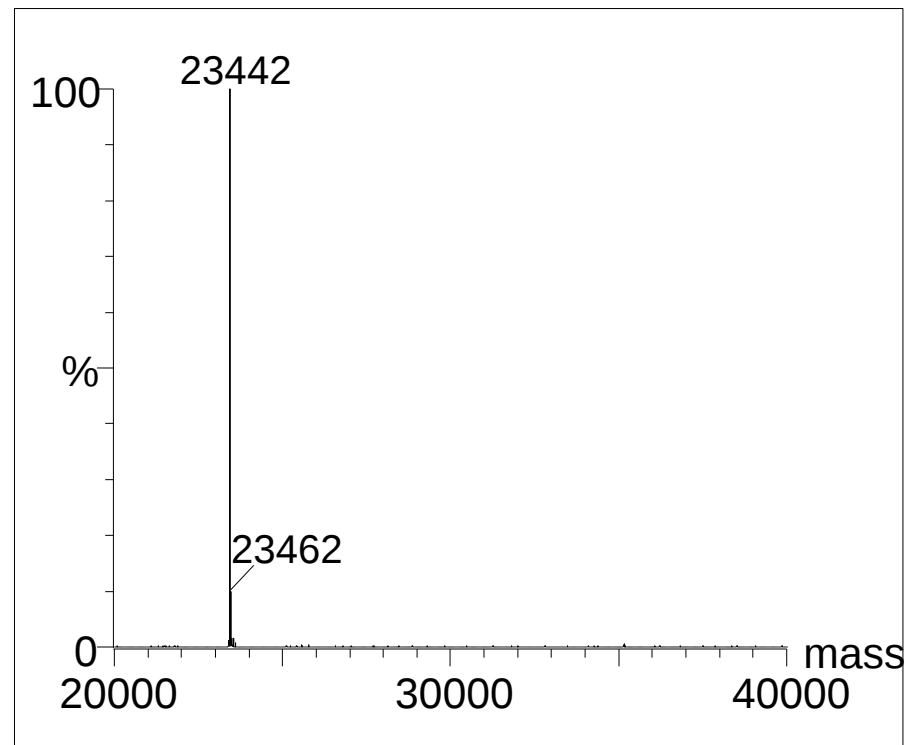
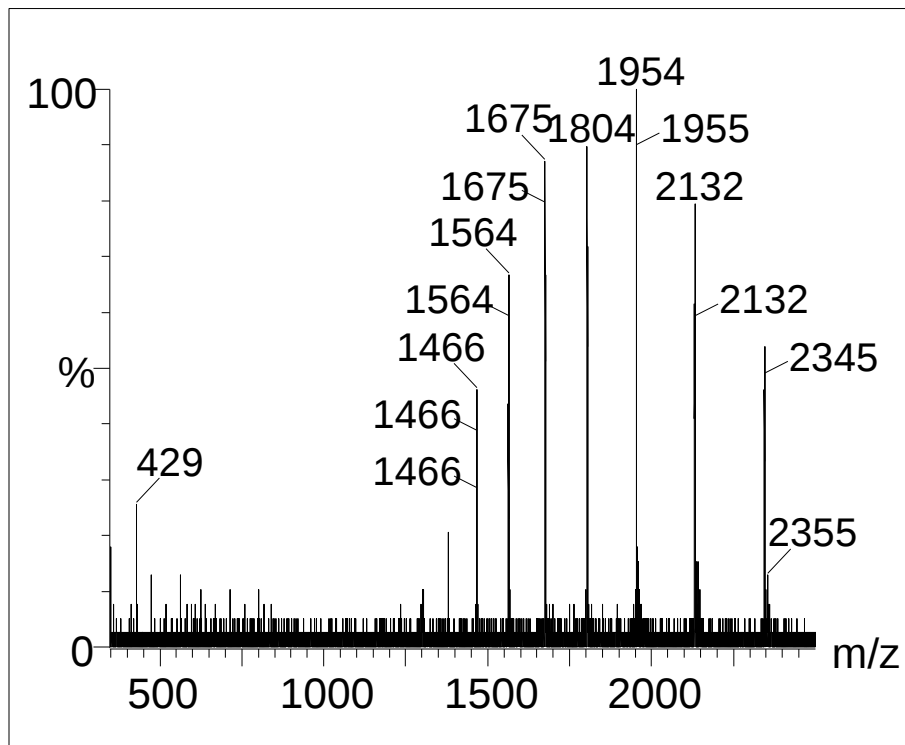
Sepax Technologies

www.sepax-tech.com

Better Surface Chemistry for Better Separation © Sepax Technologies, Inc.

Light Chain

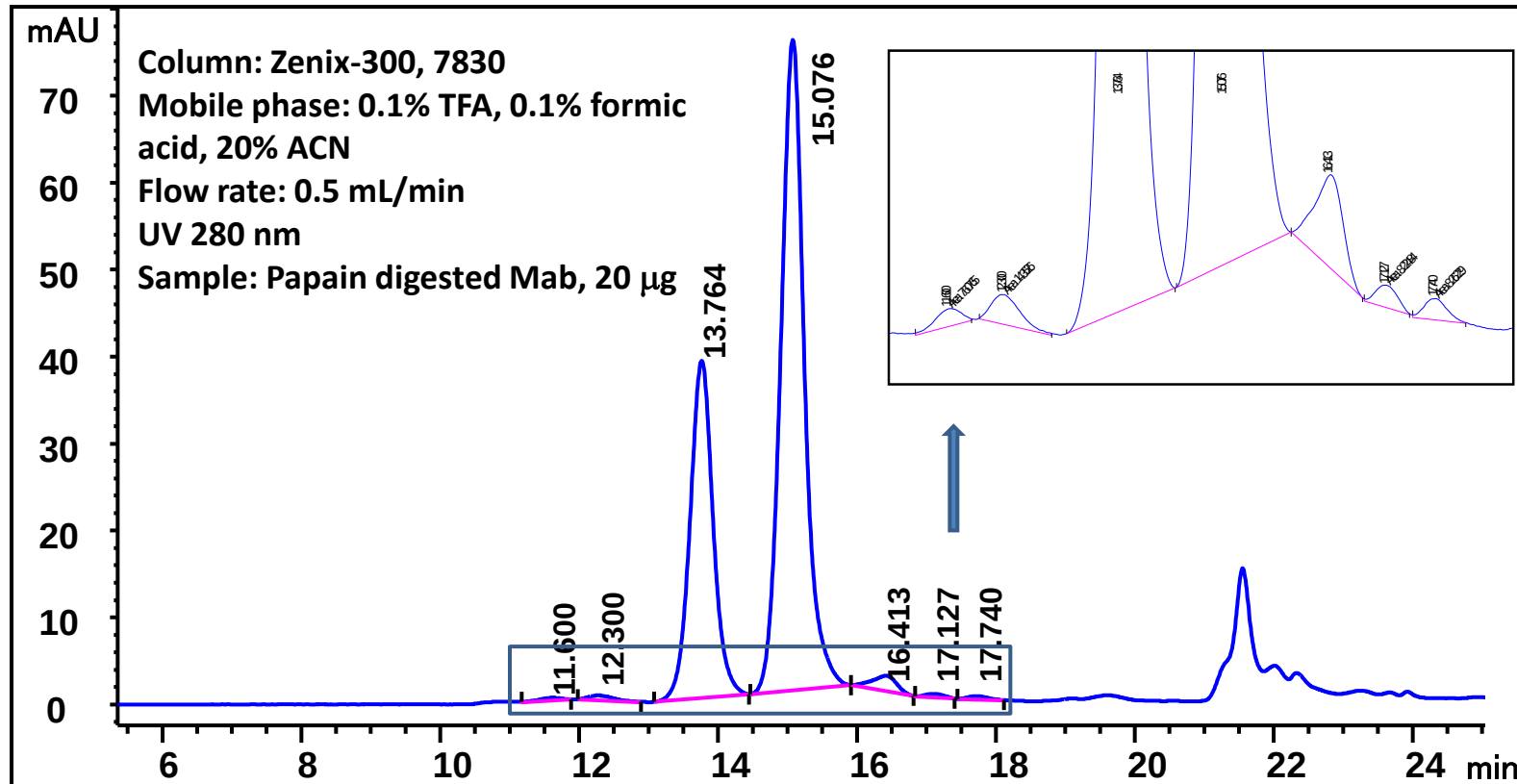
LIGHT_MAB 451 (15.266)



Fab and Fc-papain digested MAb

Fab and Fc separation on Zenix-300 7.8 x 300 mm

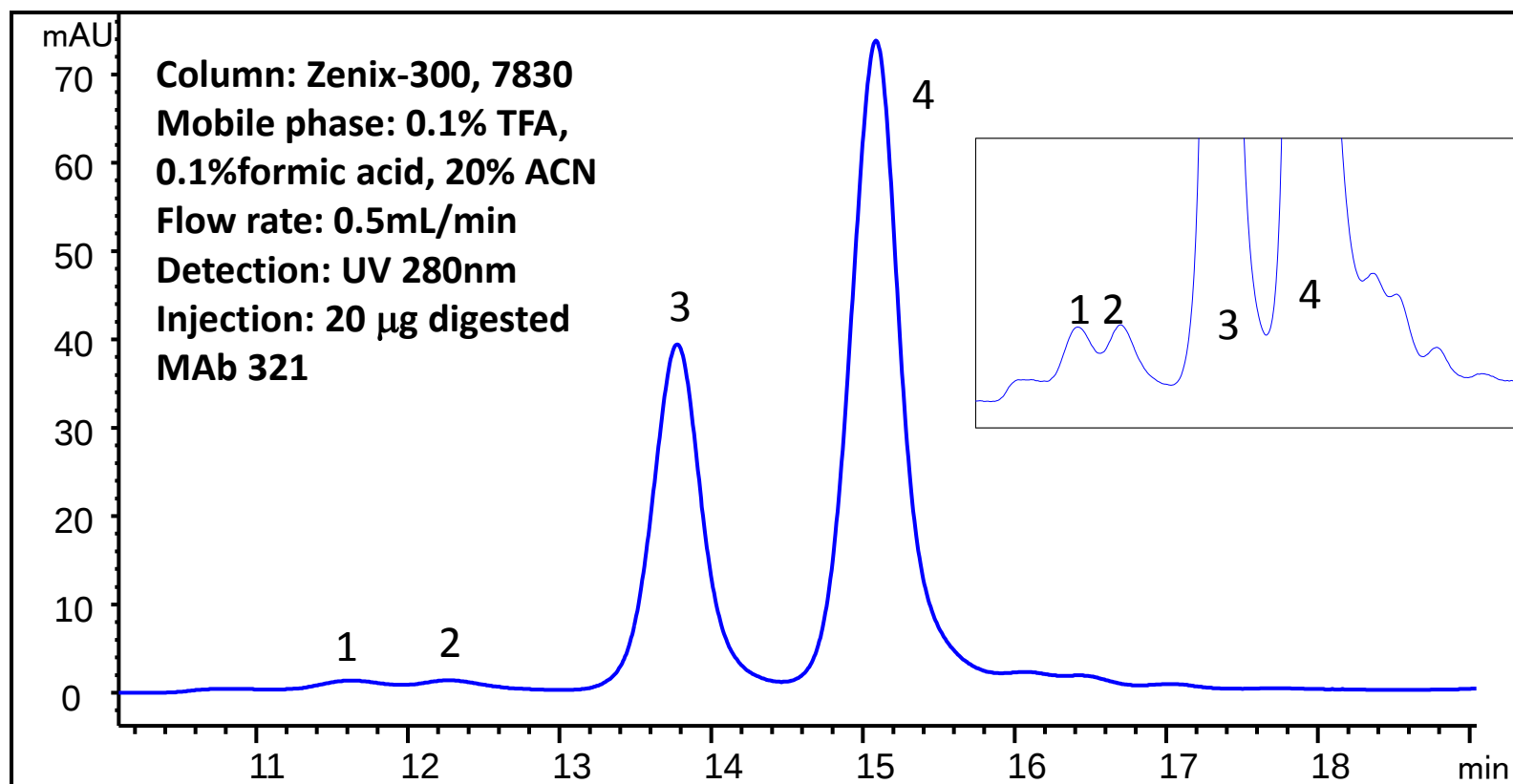
Papain digestion: 2mM EDTA, 5mM cysteine, 0.1 M Tris-HCl, MAb: papain=100:1, 37 °C for 3 hours.



#	Time	Area	Height	Width	Area%
1 Intact Mab	11.6	7.6	3.3E-1	0.3818	0.277
2 Fab+Fc	12.3	14.4	5.7E-1	0.4218	0.522
3 Fc	13.764	897.8	38.8	0.3519	32.647
4 Fab	15.076	1769.9	74.8	0.3579	64.357
5	16.413	43.9	1.8	0.3534	1.598
6	17.127	8.2	4.2E-1	0.3232	0.299
7	17.74	8.3	4.1E-1	0.3399	0.300



1 mM Cysteine and 100:1, 2 hours

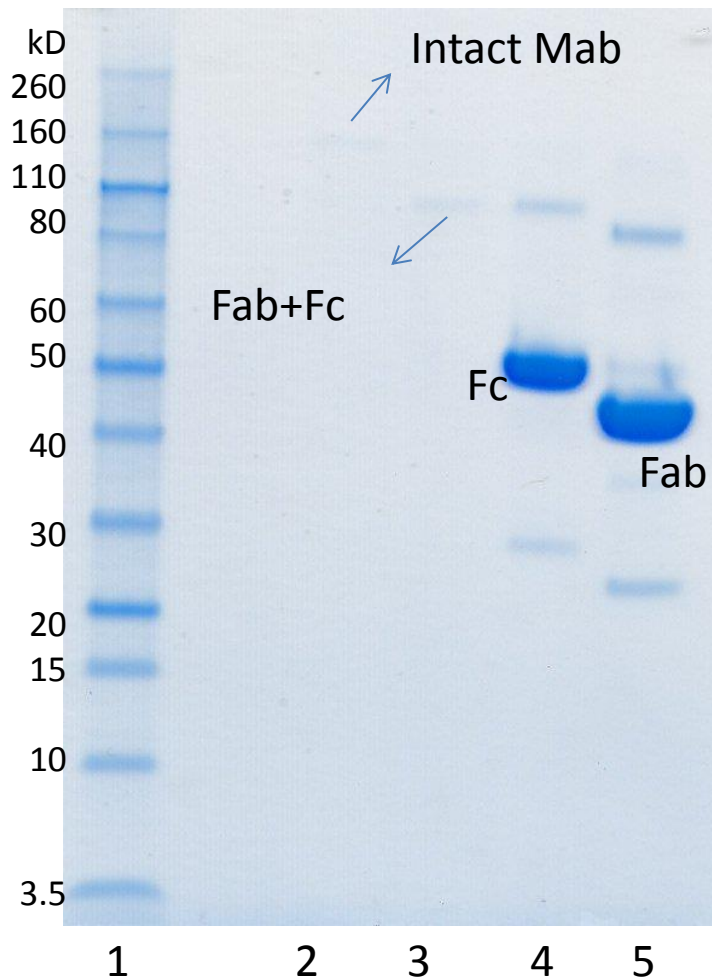


Gel picture of collected fractions from Zenix-300 7830

Fractions were collected from the runs in previous slide.

Peak 1 and 2 were pooled from two HPLC runs separately.

1. Marker
2. Peak 1 Intact MAb 321
3. Peak2 Fab + Fc
4. Peak 3 Fc
5. Peak 4 Fab

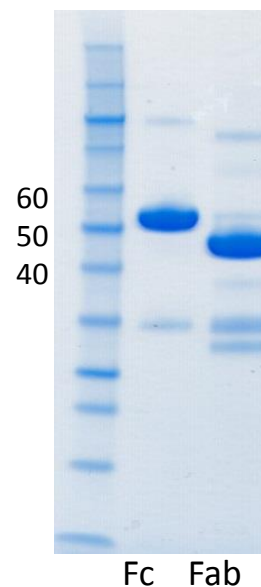
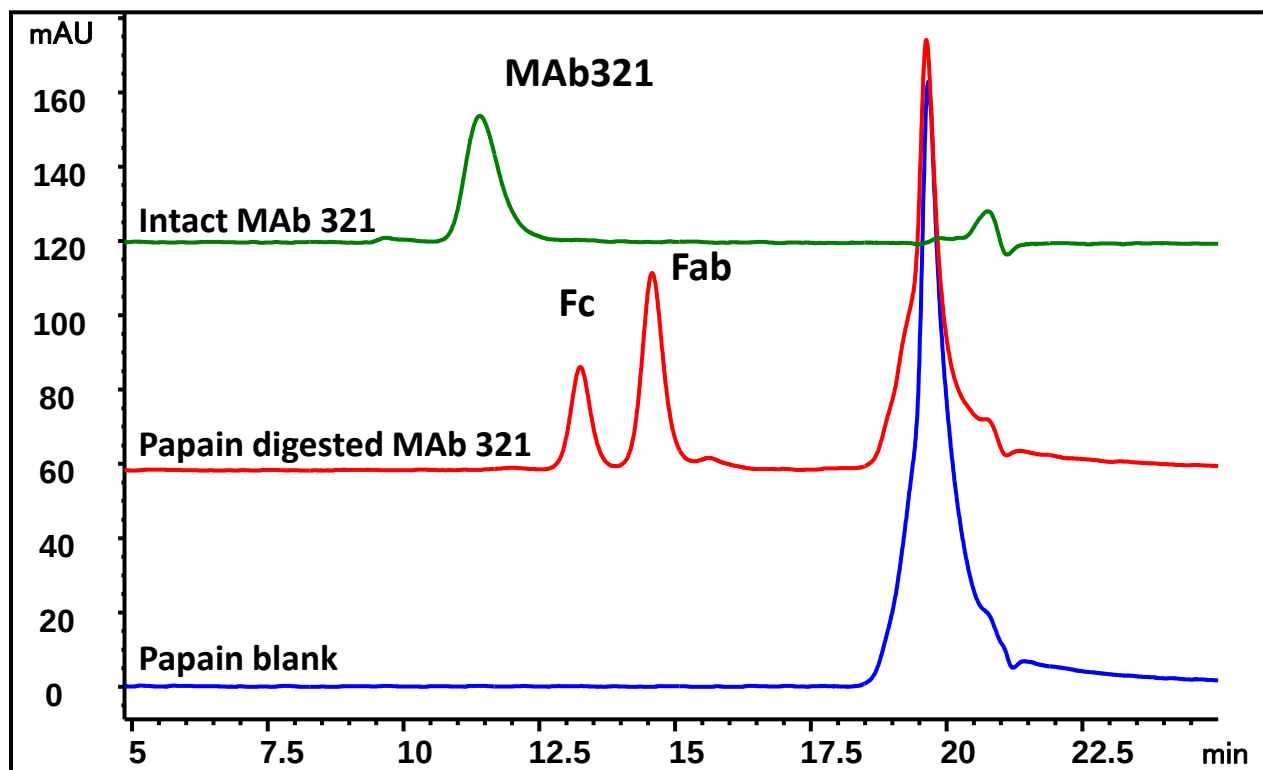


Fab/Fc separation on Zenix-300 4630

Column: Zenix-300, 4630, Mobile phase: 0.1% TFA, 0.1% formic acid, 20% ACN

Flow rate: 0.2 mL/min, injection: 5 µg intact MAb 321, 5 µg papain digested MAb 321

Papain digestion: 2mM EDTA, 5mM cysteine, 0.1 M Tris-HCl, MAb: papain=100:1, 37 °C for 3.5 hours.



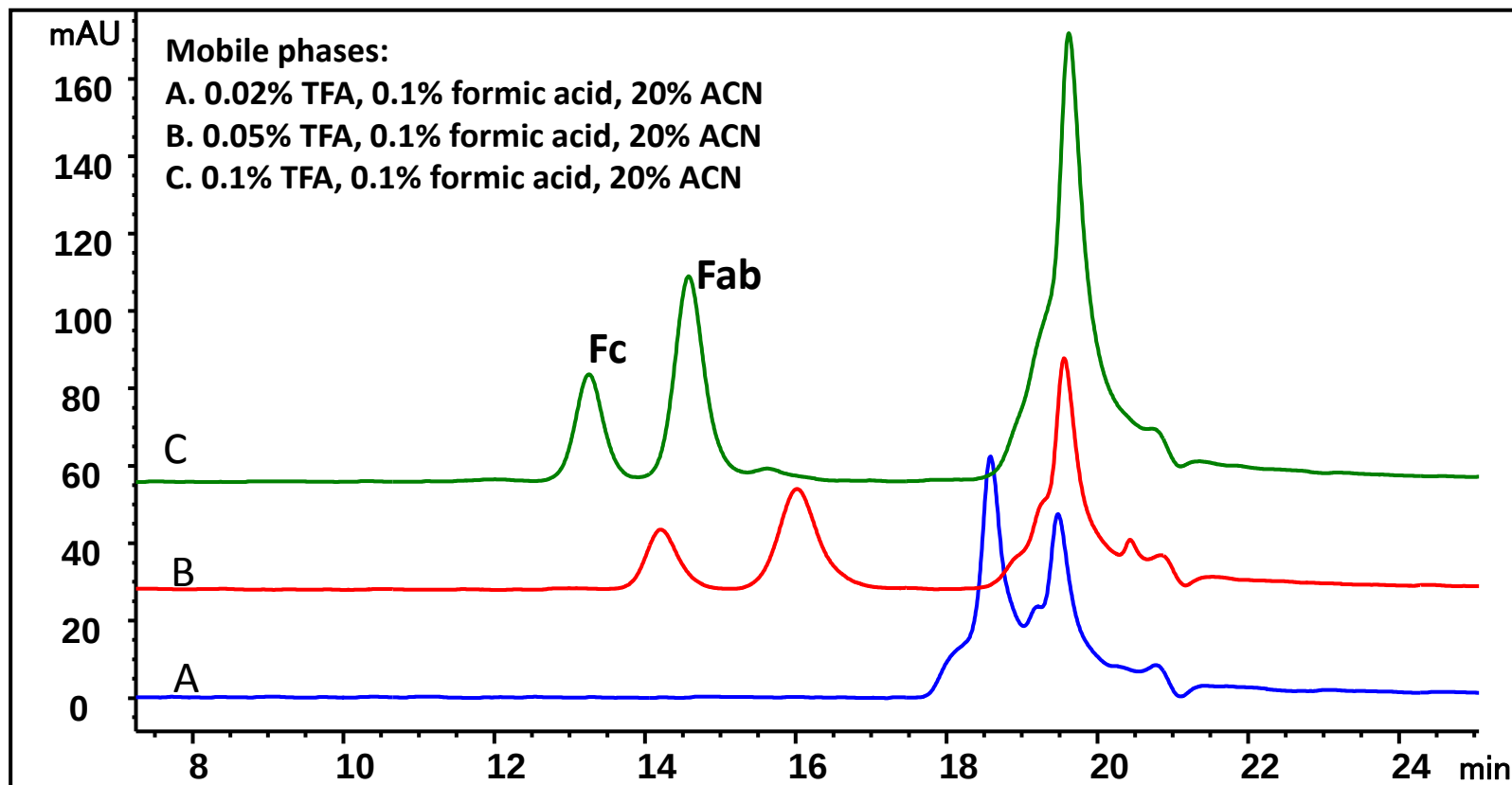
Fractions of Fc and Fab were collected from a 7.8x 300mm run with 20 µg digested MAb 321, speed-vac dried and run on 4-12% Bis-tris gel.

Different TFA concentration effect on Papain digested MAb (Fab/Fc) separation on Zenix-300 4630

Column: Zenix-300, 4630, mobile phase: as indicated

Flow rate: 0.2 mL/min, injection: 5 μ g Papain digested MAb 321

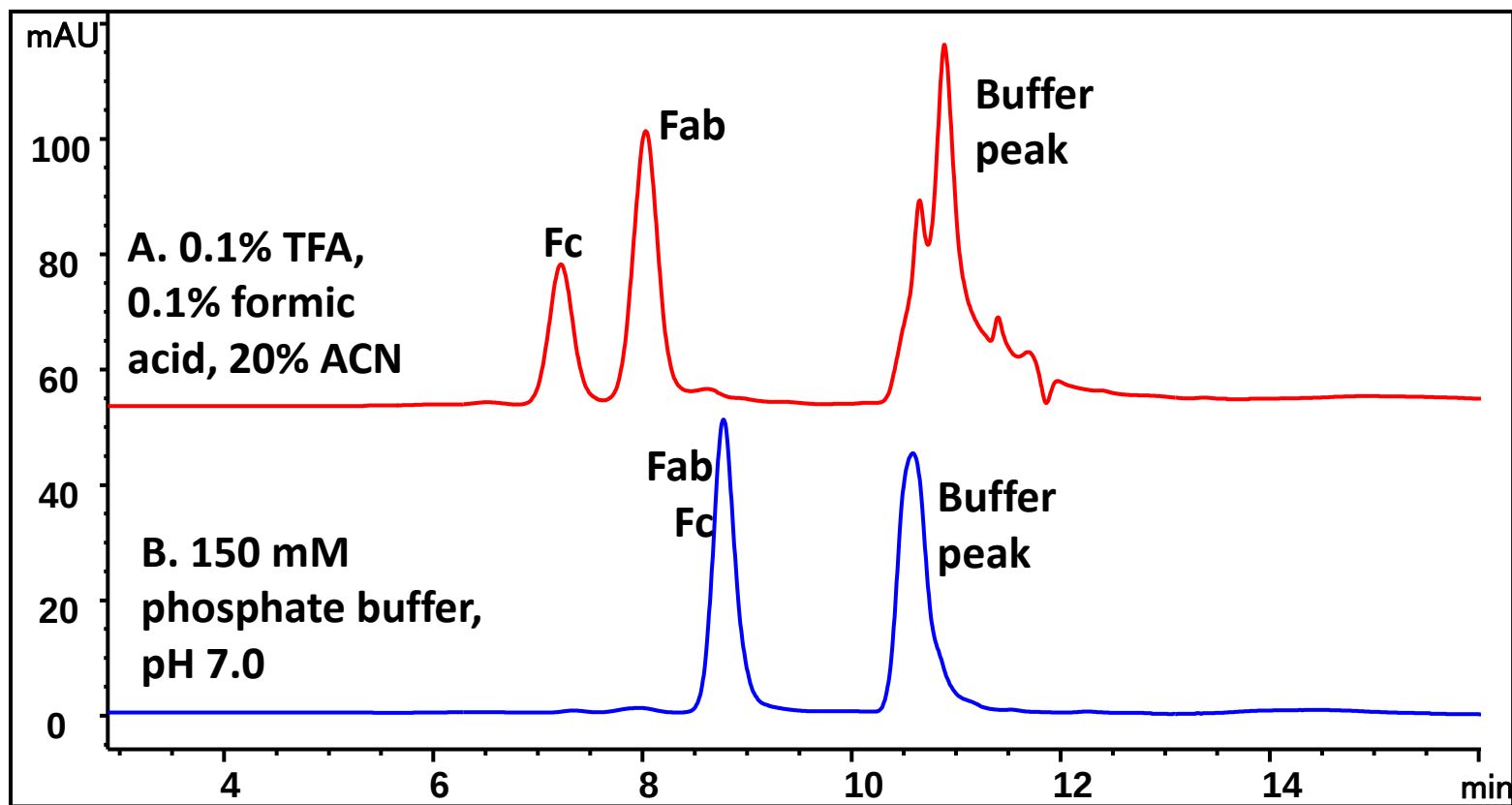
Papain digestion: 2mM EDTA, 5 mM Cysteine, 100 mM Tris-HCl, pH 7.6, MAb 1mg/mL, 3.5 hours incubation at 37 $^{\circ}$ C.



Fab and Fc separation on Zenix-300 4630, with salt and volatile buffer

Papain digestion: 2mM EDTA, 5 mM Cysteine, 100 mM Tris-HCl, pH 7.6, MAb 1mg/mL, 3.5 hours incubation at 37 °C

Column: Zenix-300 4.6x300 mm, **flow: 0.35 mL/min**, injection: 10 µL for A, 5 µL for B



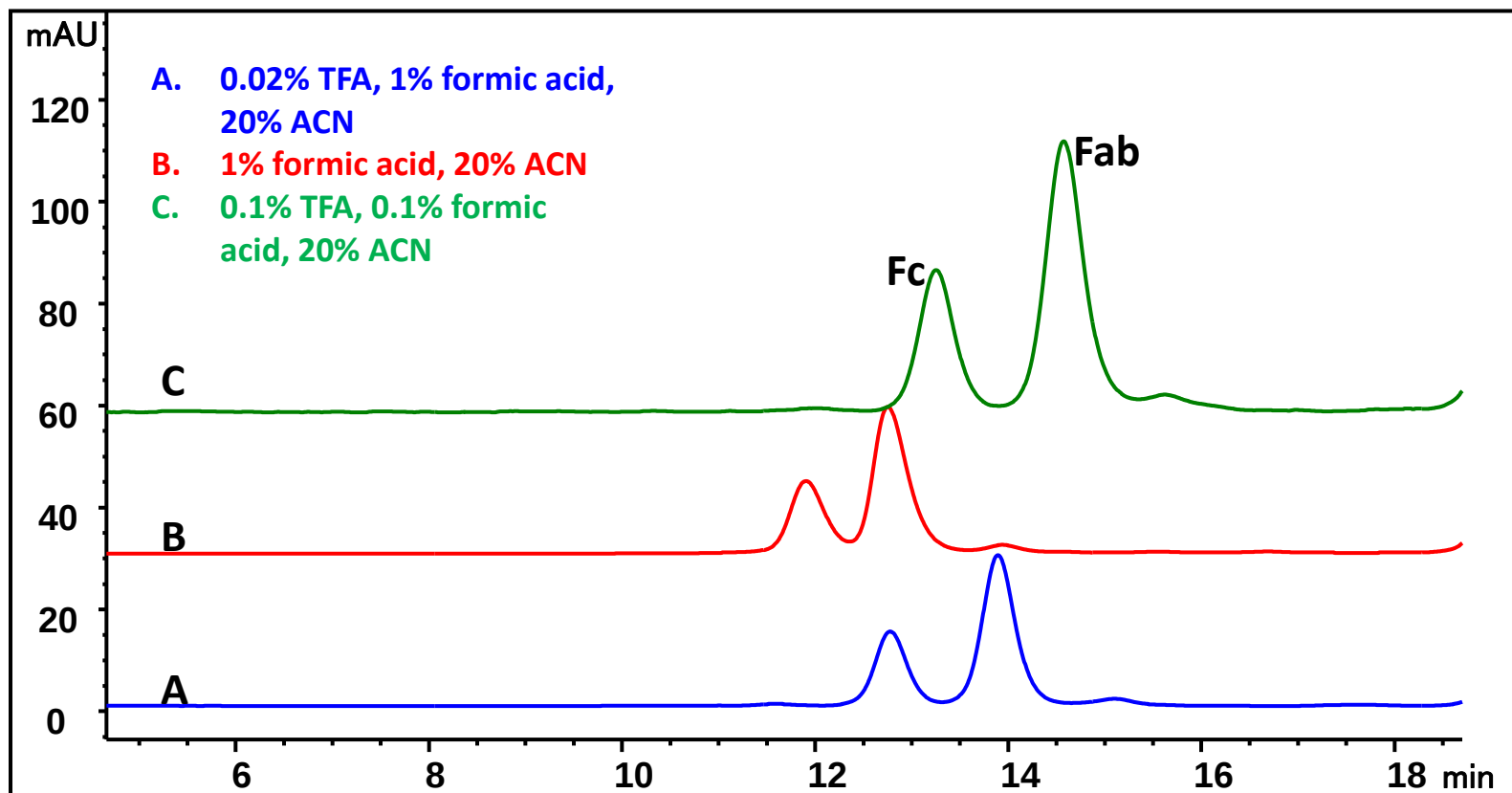
Conclusion: volatile buffer system separates Fc and Fab.

Fab and Fc separation-TFA/formic acid

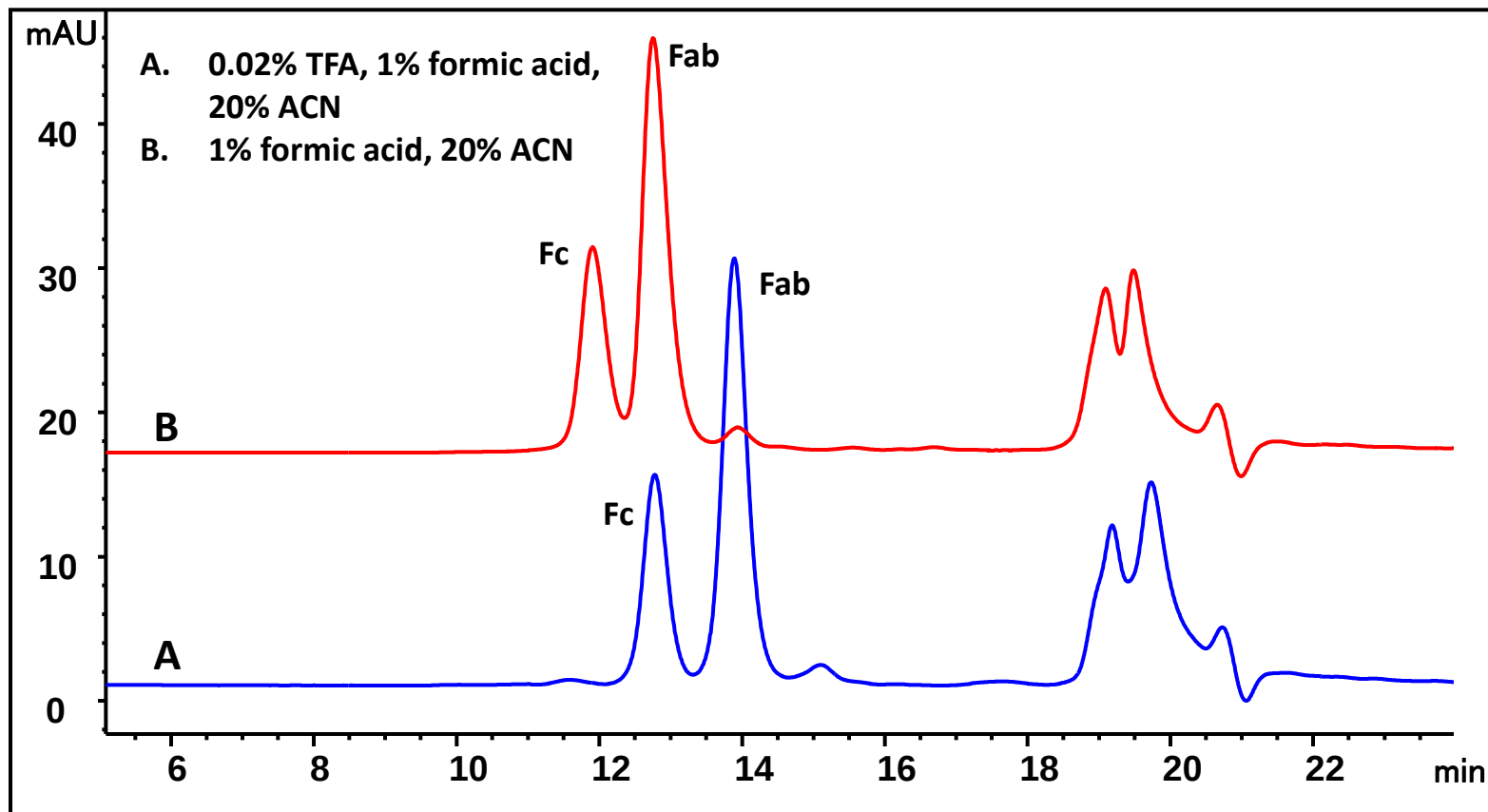
Column: Zenix-300, 4630, mobile phase: as indicated

Flow rate: 0.2 mL/min, injection: 5 μ g Papain digested MAb 321

Papain digestion: 2mM EDTA, 5 mM Cysteine, 100 mM Tris-HCl, pH 7.6, MAb 1mg/mL, 3.5 hours incubation at 37 °C.



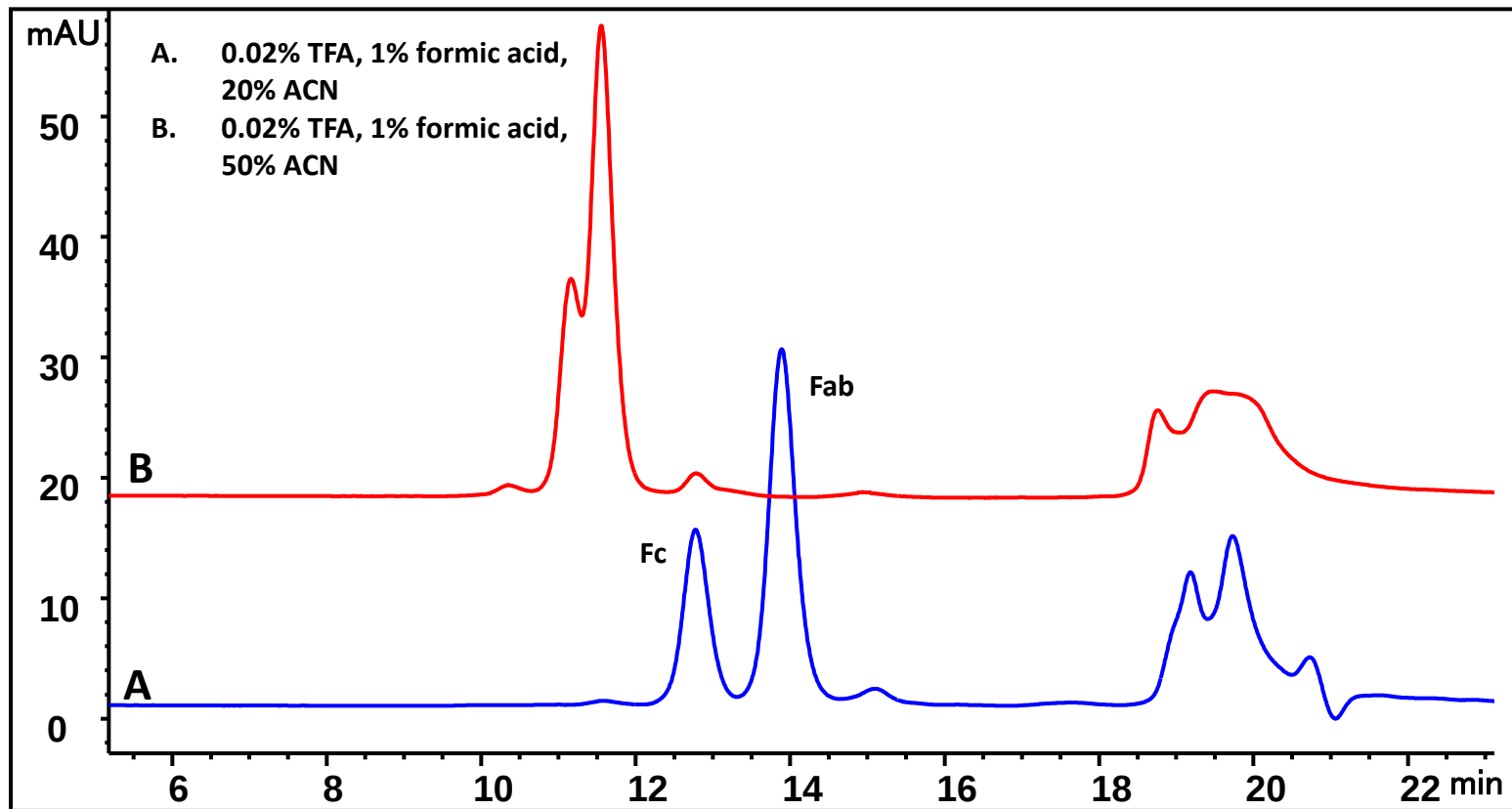
TFA affect in 1% formic acid, 20% acetonitrile- Fab/Fc separation



Dependence of column performance on mobile phase compositions

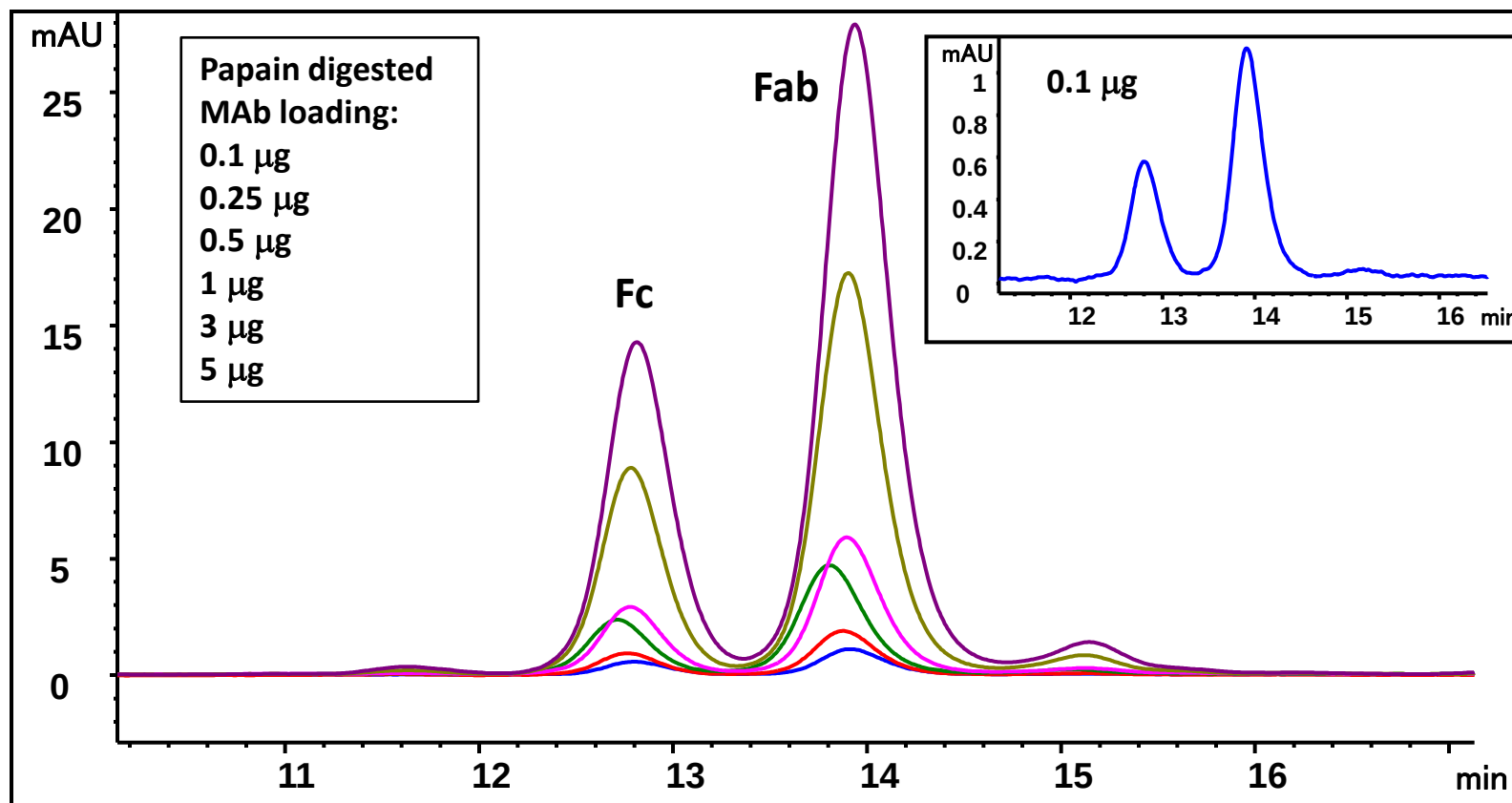
Peak	Mobile phase	RT (min)	Plate	Tailing	Resolution
Fc	0.02% TFA, 1% formic acid, 20% ACN	12.78	6824	1.09	
	1% formic acid, 20% ACN	11.91	4909	1.05	
	0.1% TFA, 0.1% formic acid, 20% ACN	13.25	5884	1.08	
Fab	0.02% TFA, 1% formic acid, 20% ACN	13.89	7551	1.09	1.77
	1% formic acid, 20% ACN	12.75	5824	1.55	1.26
	0.1% TFA, 0.1% formic acid, 20% ACN	14.58	6322	1.19	1.85

20% and 50% acetonitrile effect on Fab/Fc



Papain digested MAb loading test

Column: Zenix-300 4.6x300 mm, flow: 0.2 mL/min, mobile phase: 0.02% TFA, 1% formic acid and 20% ACN, injection: as indicated

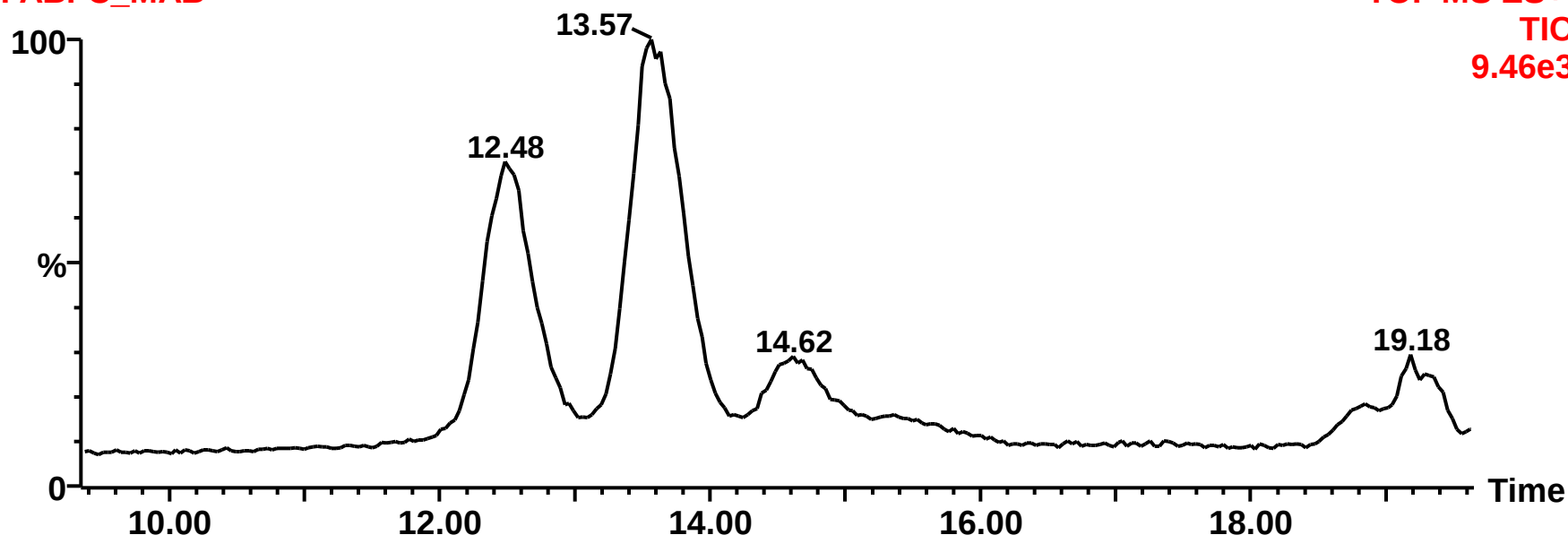


Fab and Fc-TIC

Column: Zenix-300 4.6x300 mm, flow: 0.2 mL/min, mobile phase: 0.02% TFA, 1% formic acid and 20% ACN, injection: papain digested MAb 10 µg,

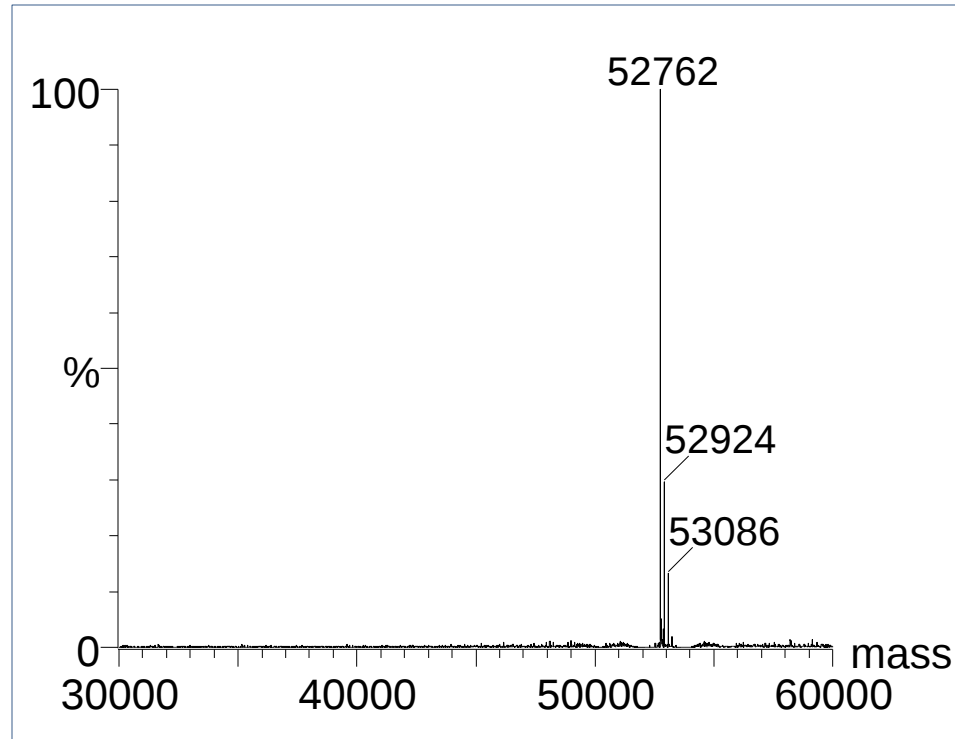
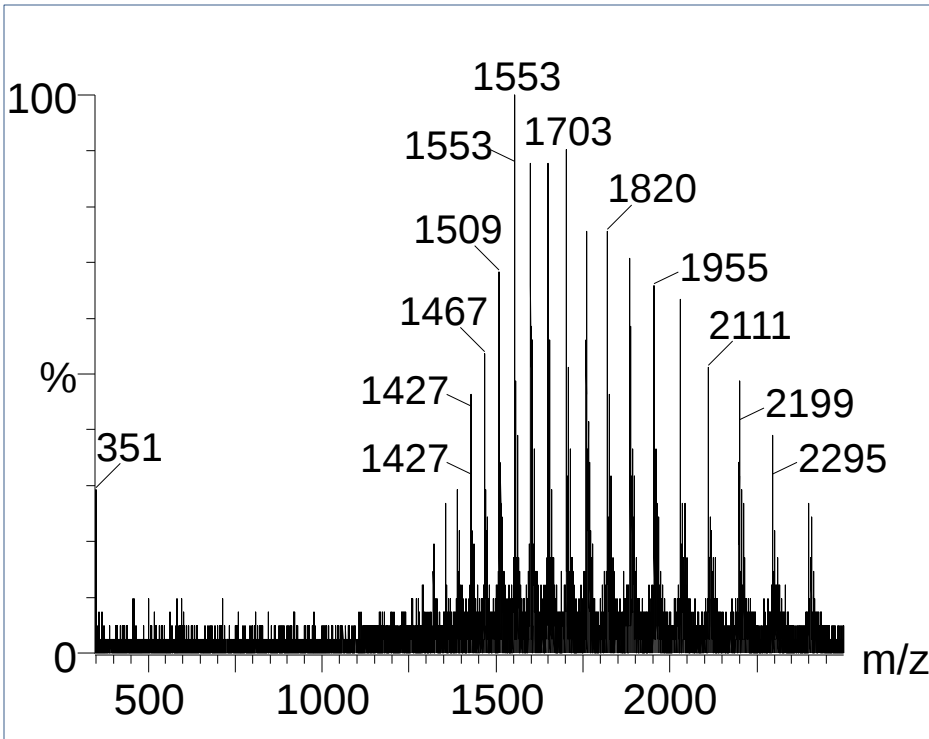
FABFC_MAB

TOF MS ES+
TIC
9.46e3



Fc Peak

FC_MAB 369 (12.484)



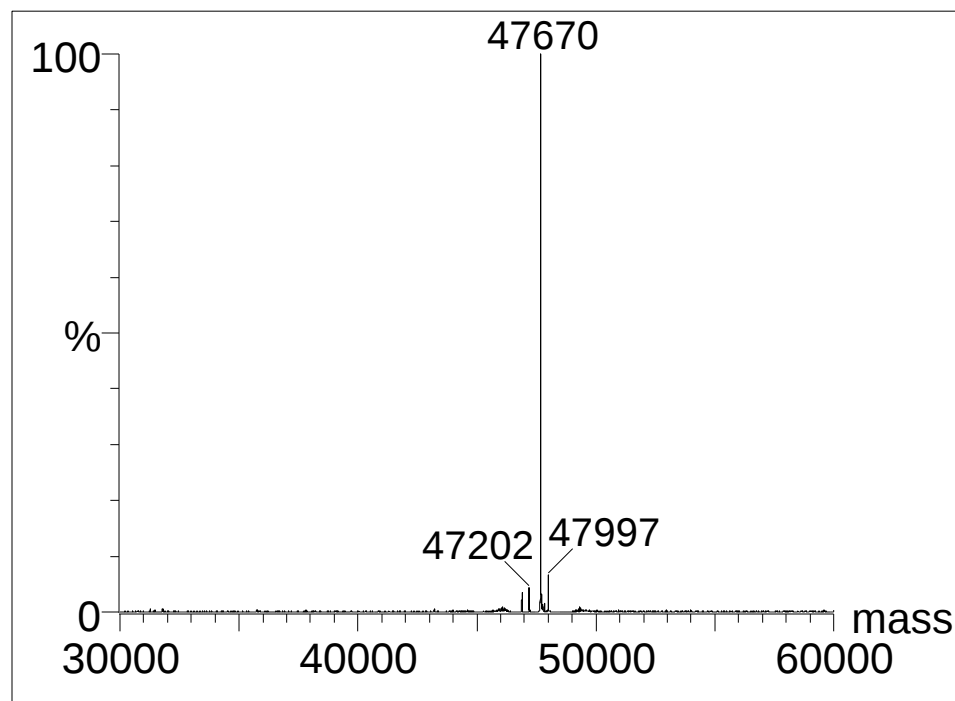
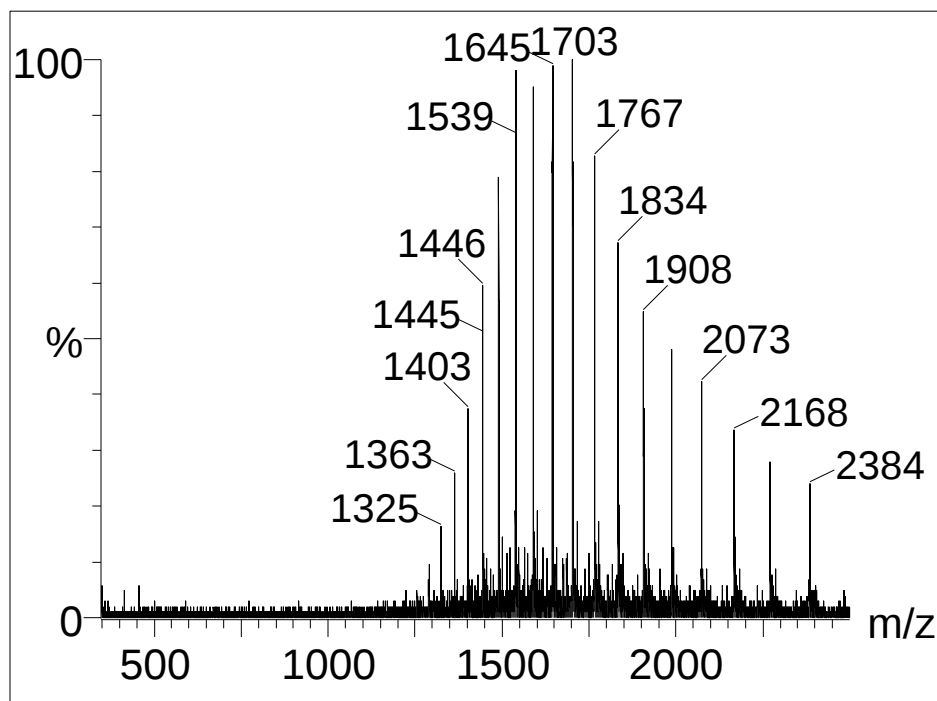
53086 Da: 2x 162 (galactose), on each heavy chain

52924 Da: 1 x 162 (galactose)



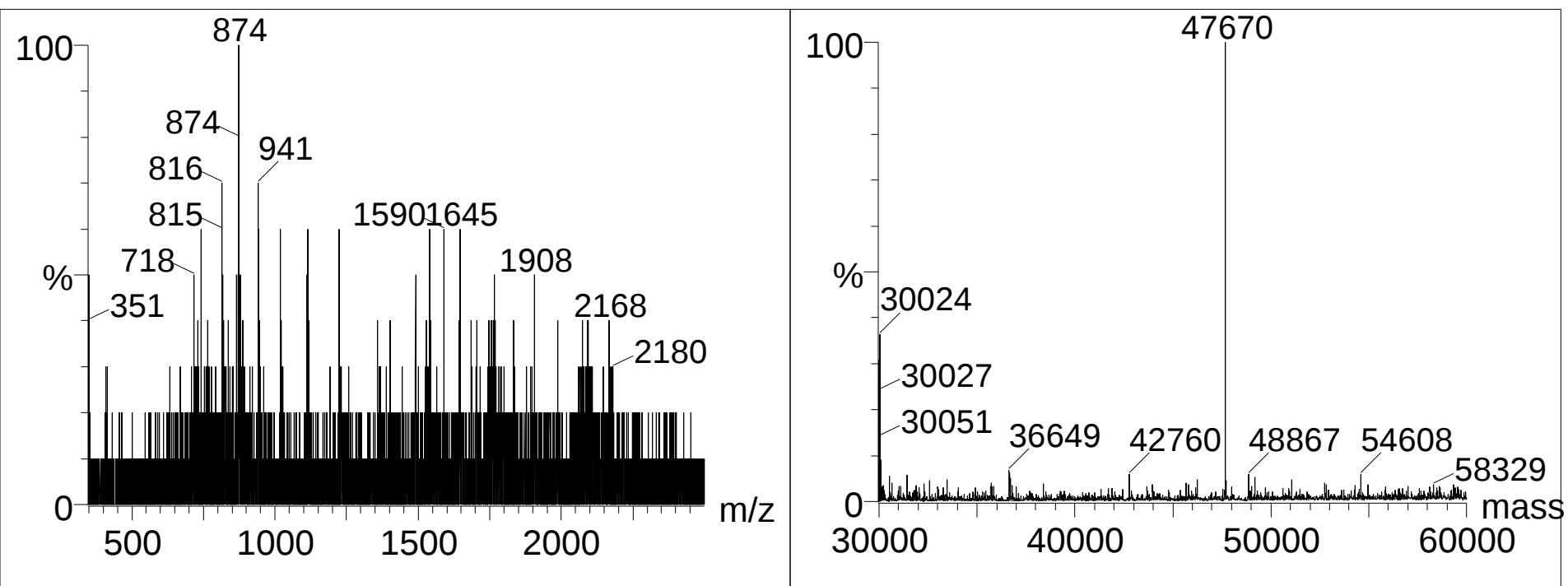
Fab peak

FAB_MAB 401 (13.567)



Peak at 14.62 min

FABFC_MAB 432 (14.617)



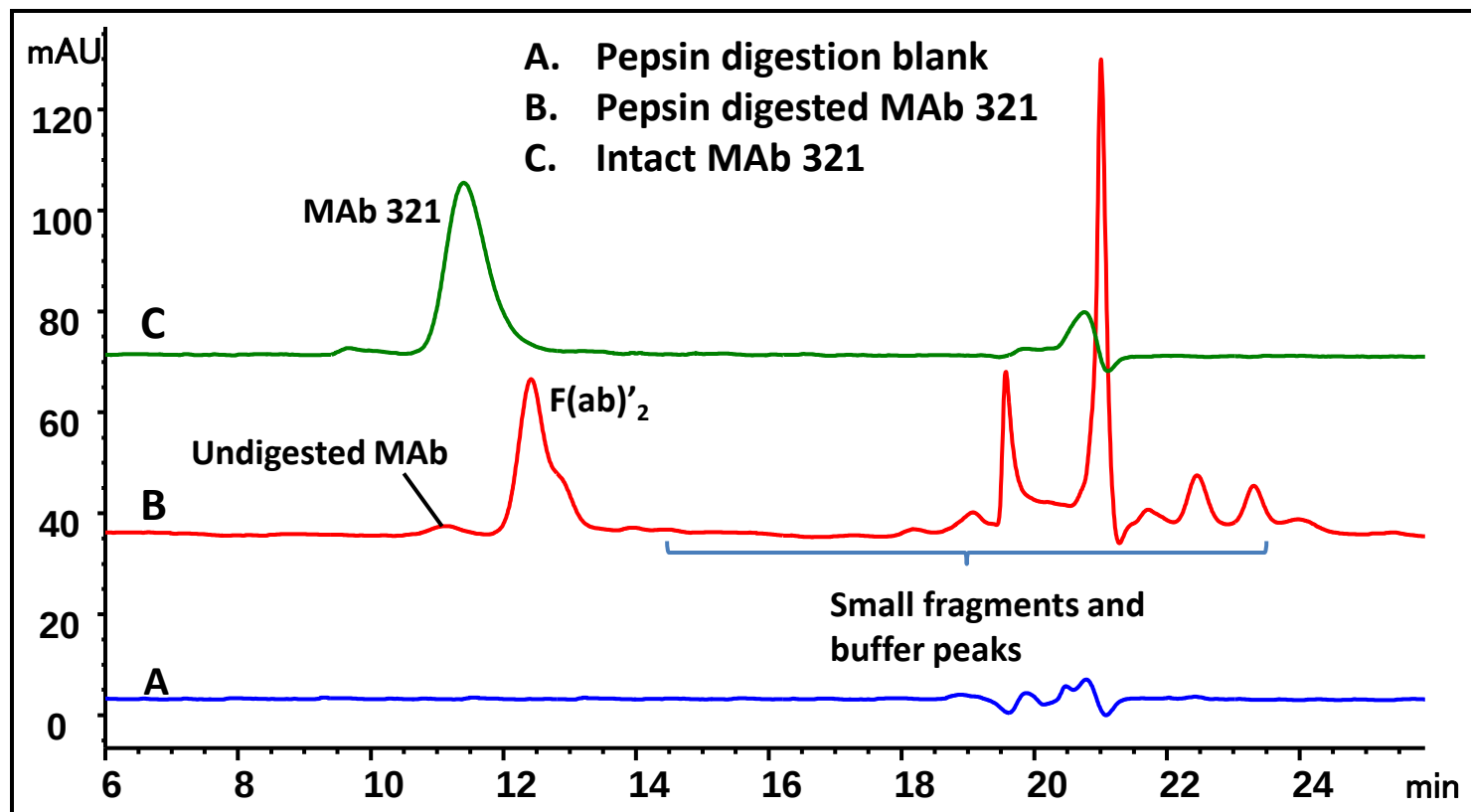
$F(ab')_2$



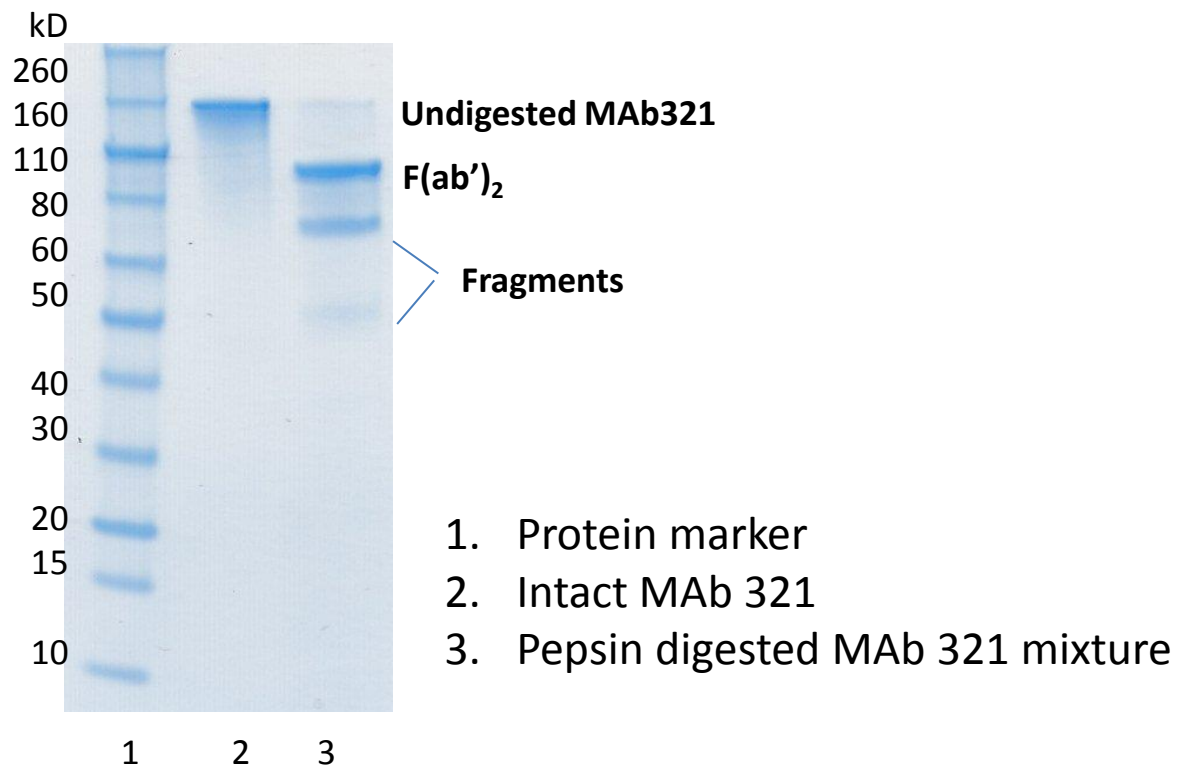
$F(ab')_2$ separation on Zenix-300 with volatile buffer

Pepsin digestion: MAb 1mg/mL, MAb:pepsin= 40:1, 20mM sodium acetate, pH 4.0, 15 hours digestion, quenched with 2M Tris.

Column: Zenix-300 4.6x300 mm, flow: **0.2 mL/min**, mobile phase: 0.1% TFA, 0.1% formic acid and 20% ACN, injection: intact MAb 5 μ g, 15 μ g pepsin digested MAb 321, 15 μ L pepsin digestion blank



4-12% Bis-Tris gel image on $F(ab')_2$

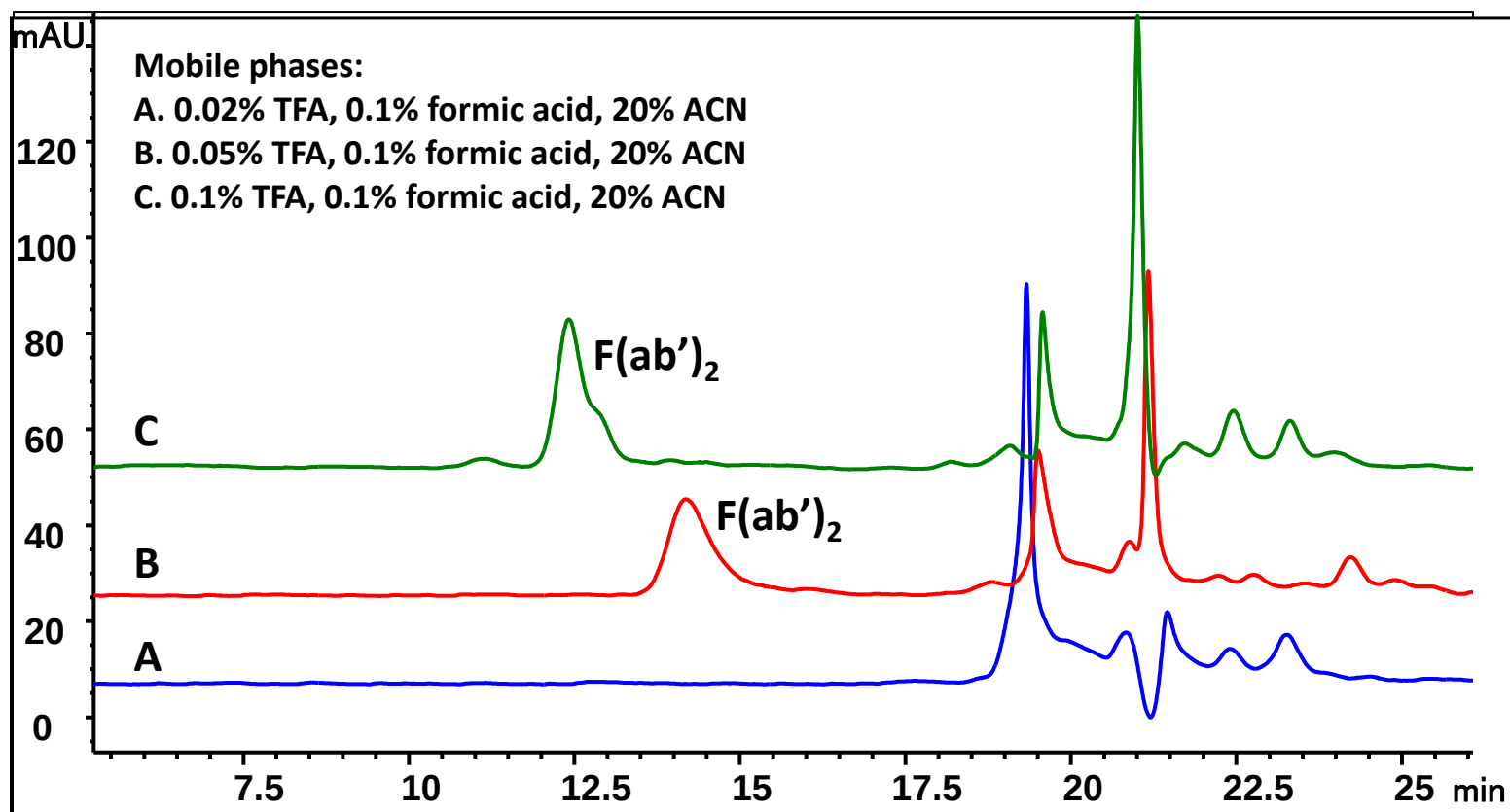


4-12% Bis-Tris gel: 5 μ g of each sample.

Different TFA concentration effect on Pepsin digested MAb-F(ab')₂ separation on Zenix-300 4630

Column: Zenix-300, 4630, mobile phase: as indicated

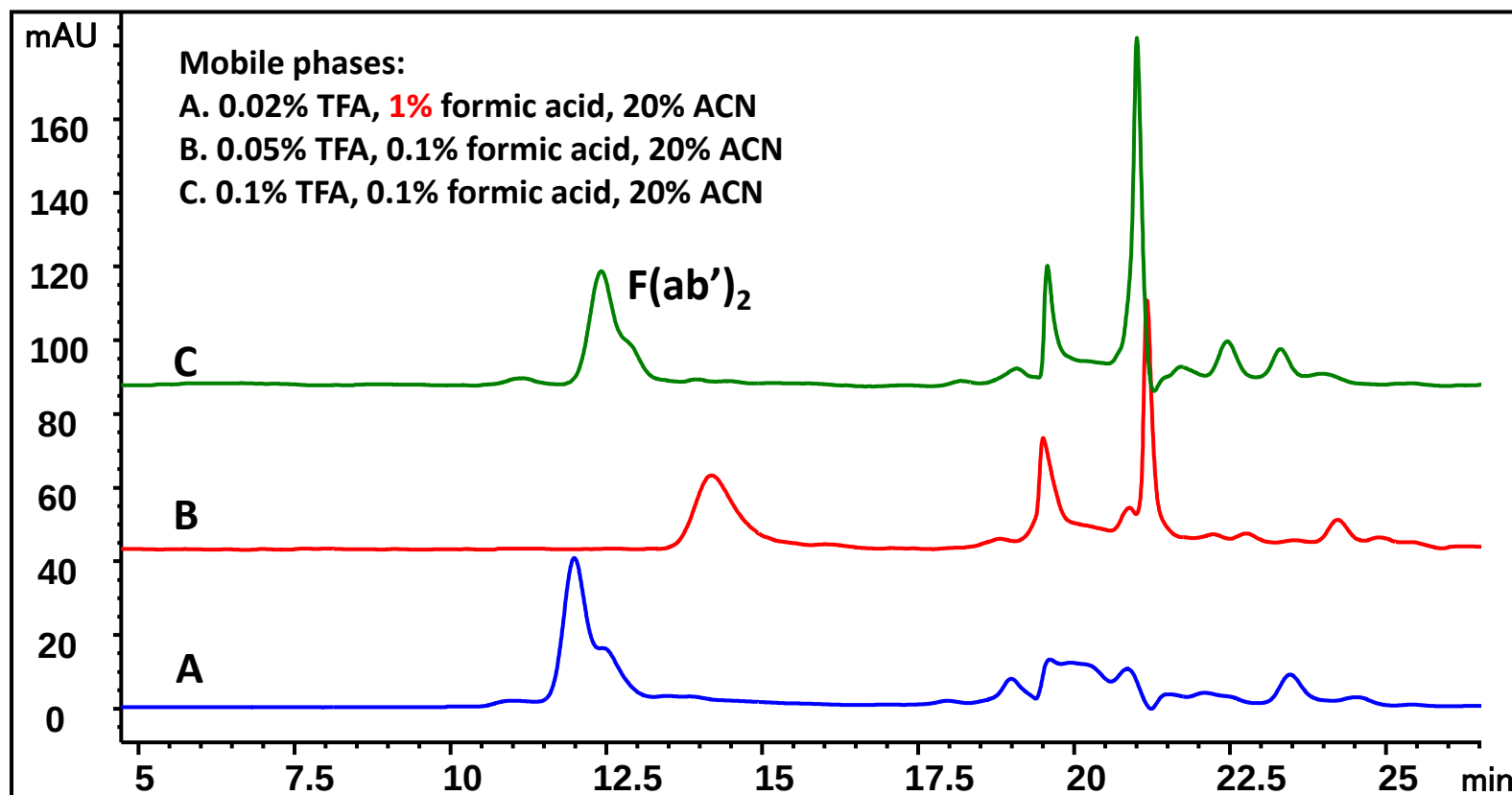
Flow rate: 0.2 mL/min, injection: 15 µg Pepsin digested MAb 321



Effect of TFA and Formic acid Concentration on Pepsin Digested MAb MAb-F(ab')₂ separation

Column: Zenix-300, 4630, mobile phase: as indicated

Flow rate: 0.2 mL/min, injection: 15 µg Pepsin digested MAb 321

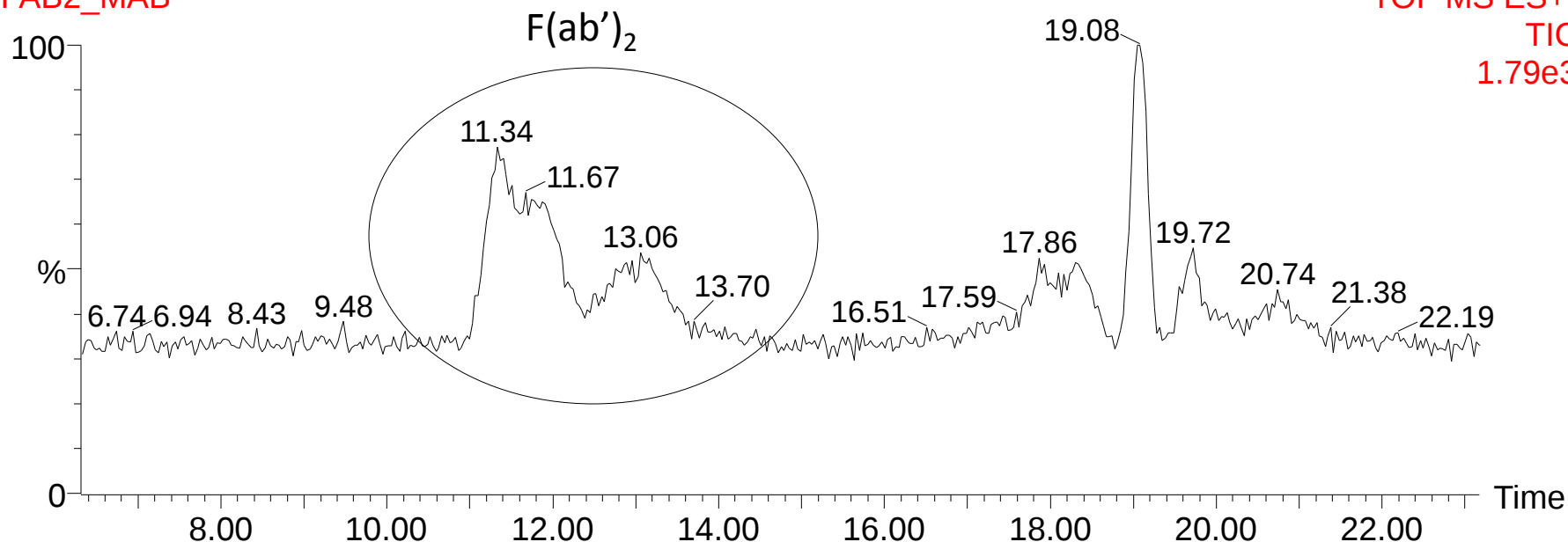


F(ab')₂ - MS TIC

Column: Zenix-300, 4630, mobile phase: 0.02% TFA, 1% formic acid and 20% ACN
Flow rate: 0.2 mL/min, injection: 8 µg Pepsin digestd MAb 321

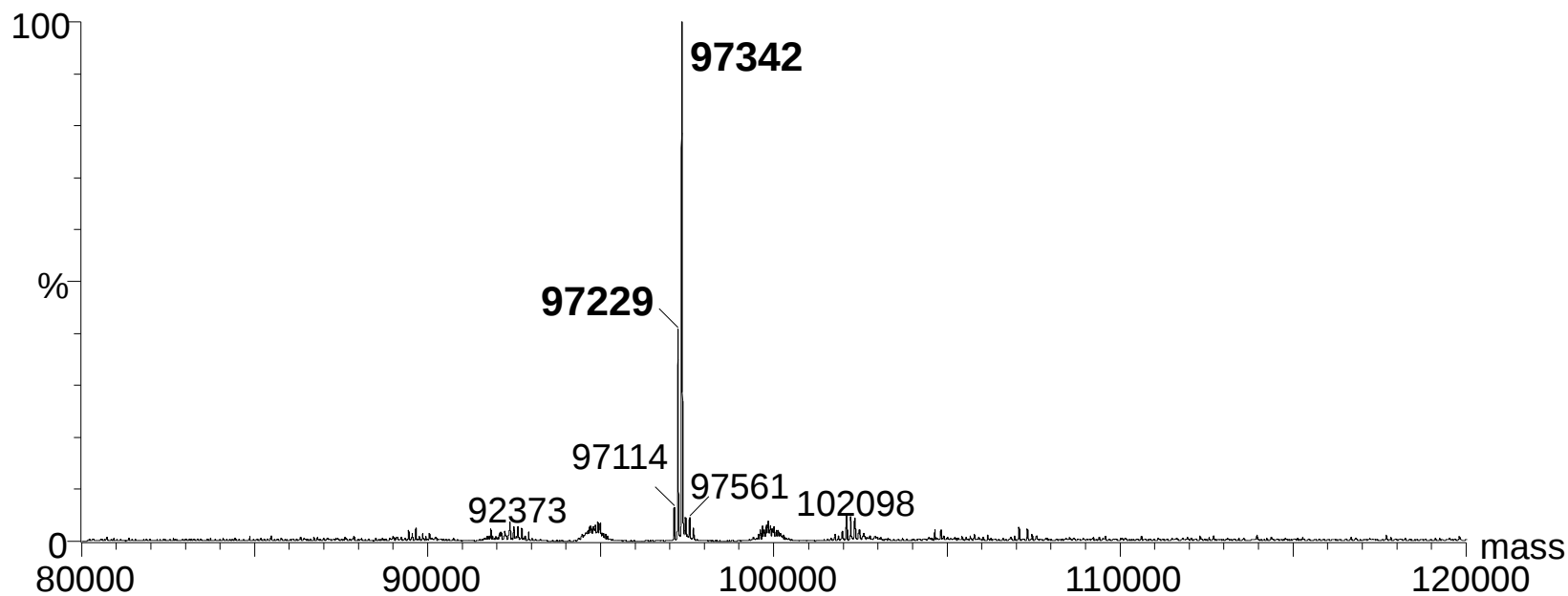
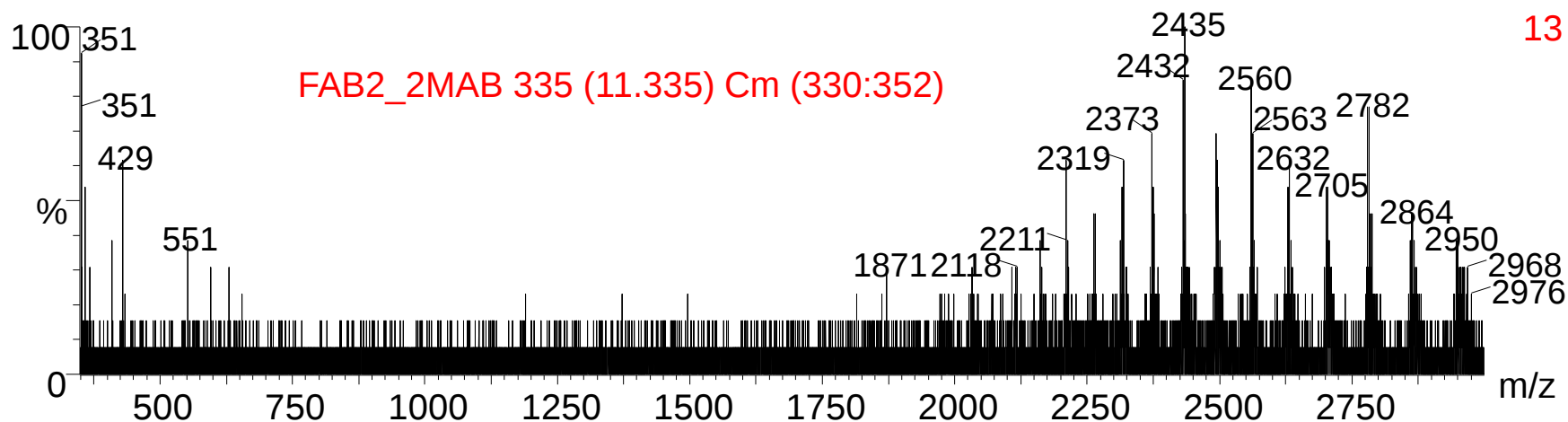
FAB2_MAB

TOF MS ES+
TIC
1.79e3



F(ab')₂-11.3 minute

TOF MS ES+



F(ab')₂-13.06 minute peak

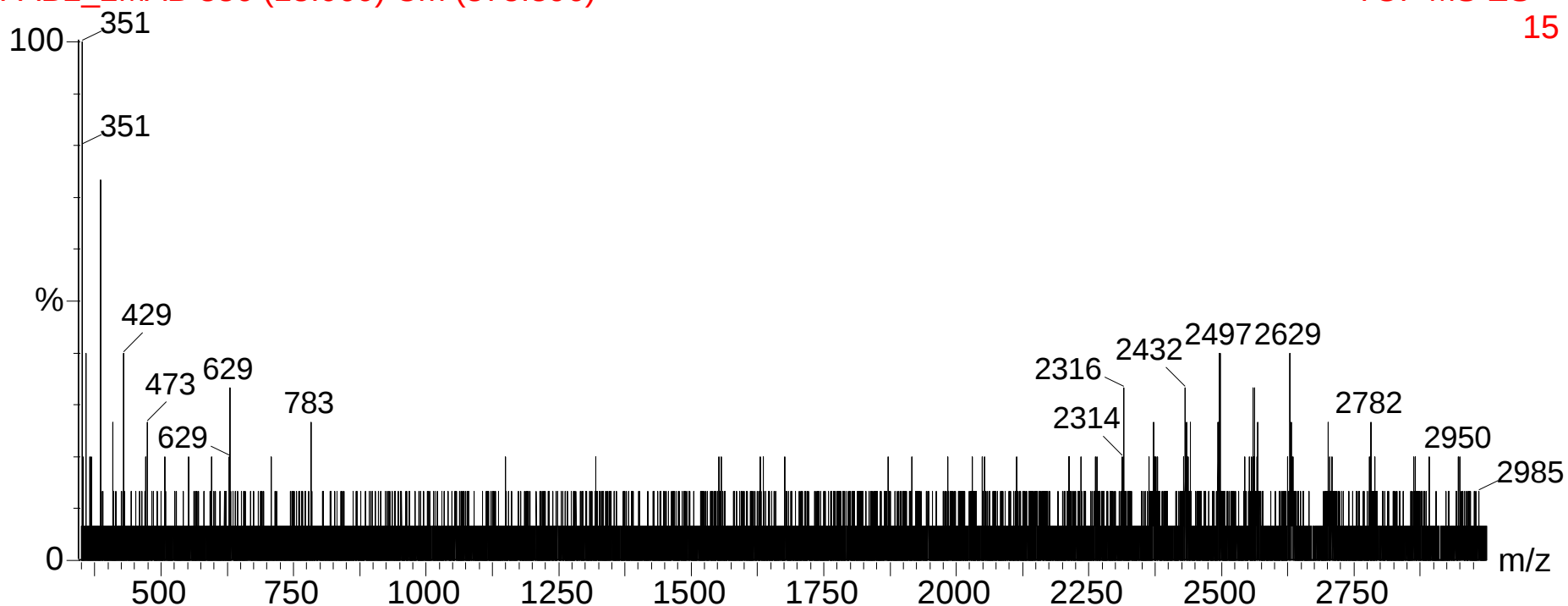
pepsin MAB

UH080
26.966291

14-Dec-2011 09:52:29

FAB2_2MAB 386 (13.060) Cm (375:396)

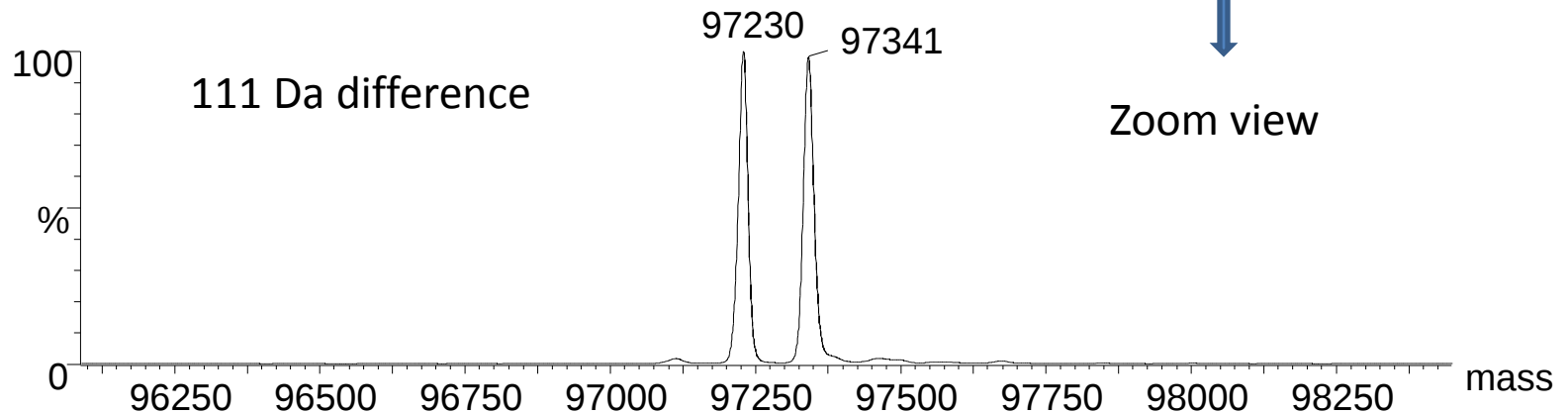
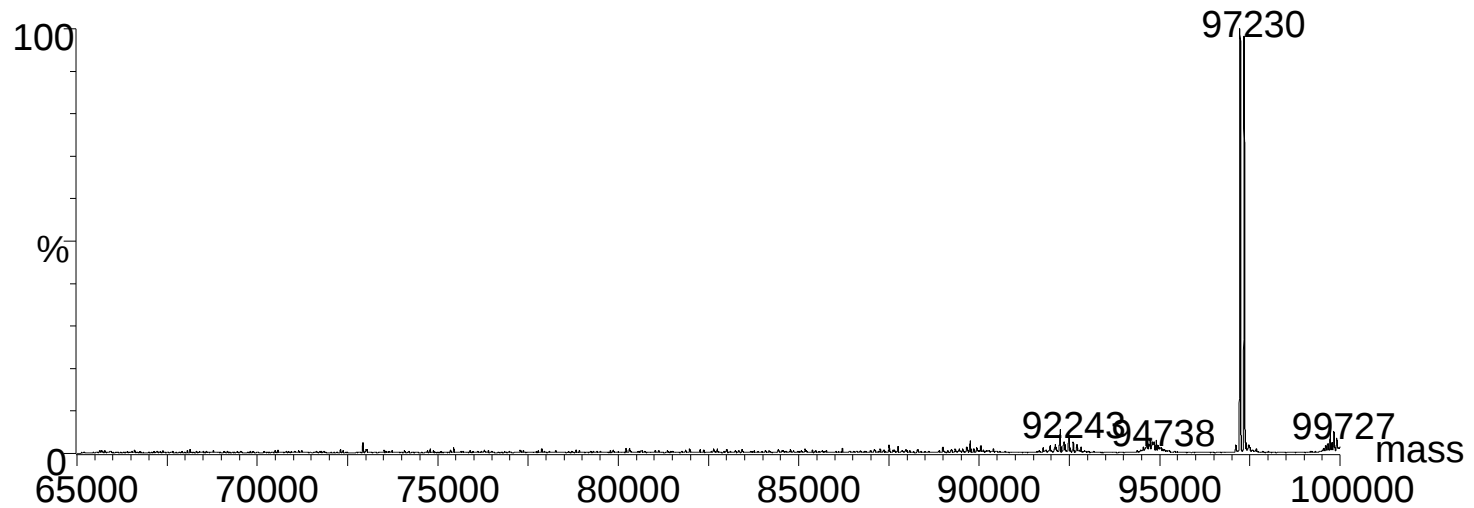
TOF MS ES+
15



F(ab')₂-13.06 minute peak

FAB2_2MAB 386 (13.060)

TOF MS ES+

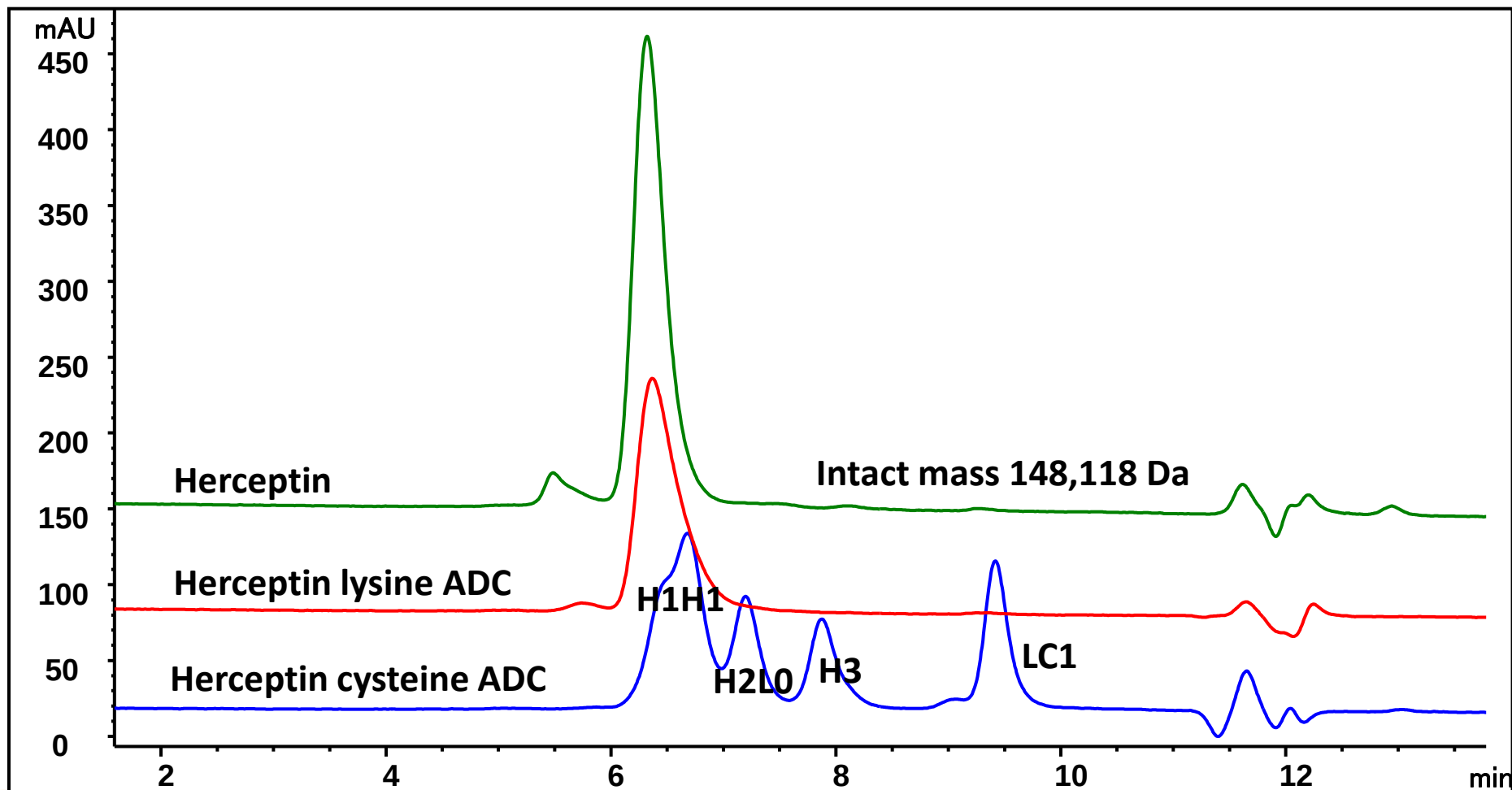


Intact Cysteine ADC and Lysine ADC separation under volatile denaturing mobile phase

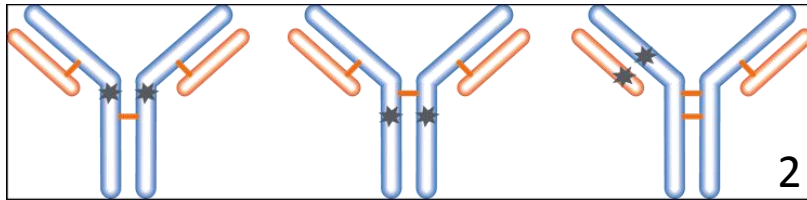
Column: Zenix-C SEC-300 (3 μm , 300 $\text{\AA}$, 4.6 x 300 mm), Mobile phase: **0.1% TFA, 0.1% formic acid and 20% ACN**

Flow rate: 0.35 mL/min; Detector: UV 214 nm; Column temperature: 25 $^{\circ}\text{C}$

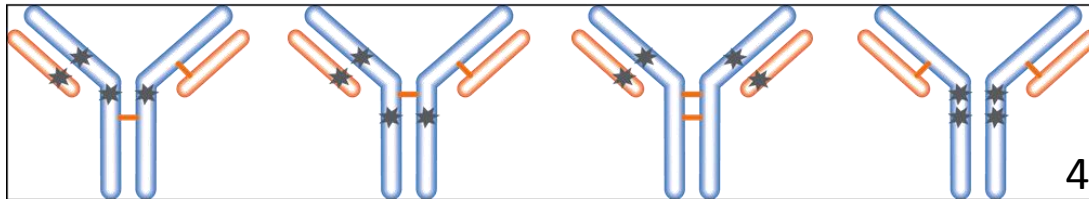
Injection volume: 2 μg , Sample: Herceptin ADCs 1mg/mL



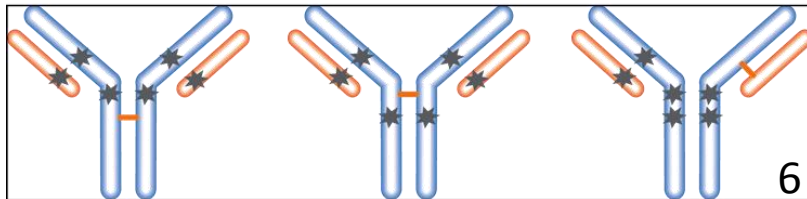
Possible Different cysteine ADC components under acidic/denaturing condition



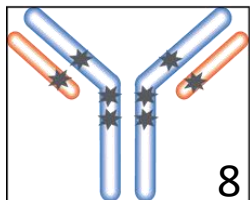
Intact 2, L1 and H1HL



L1, H2H1L, H1H1, LH2



L1, H2H2, H3, LH2

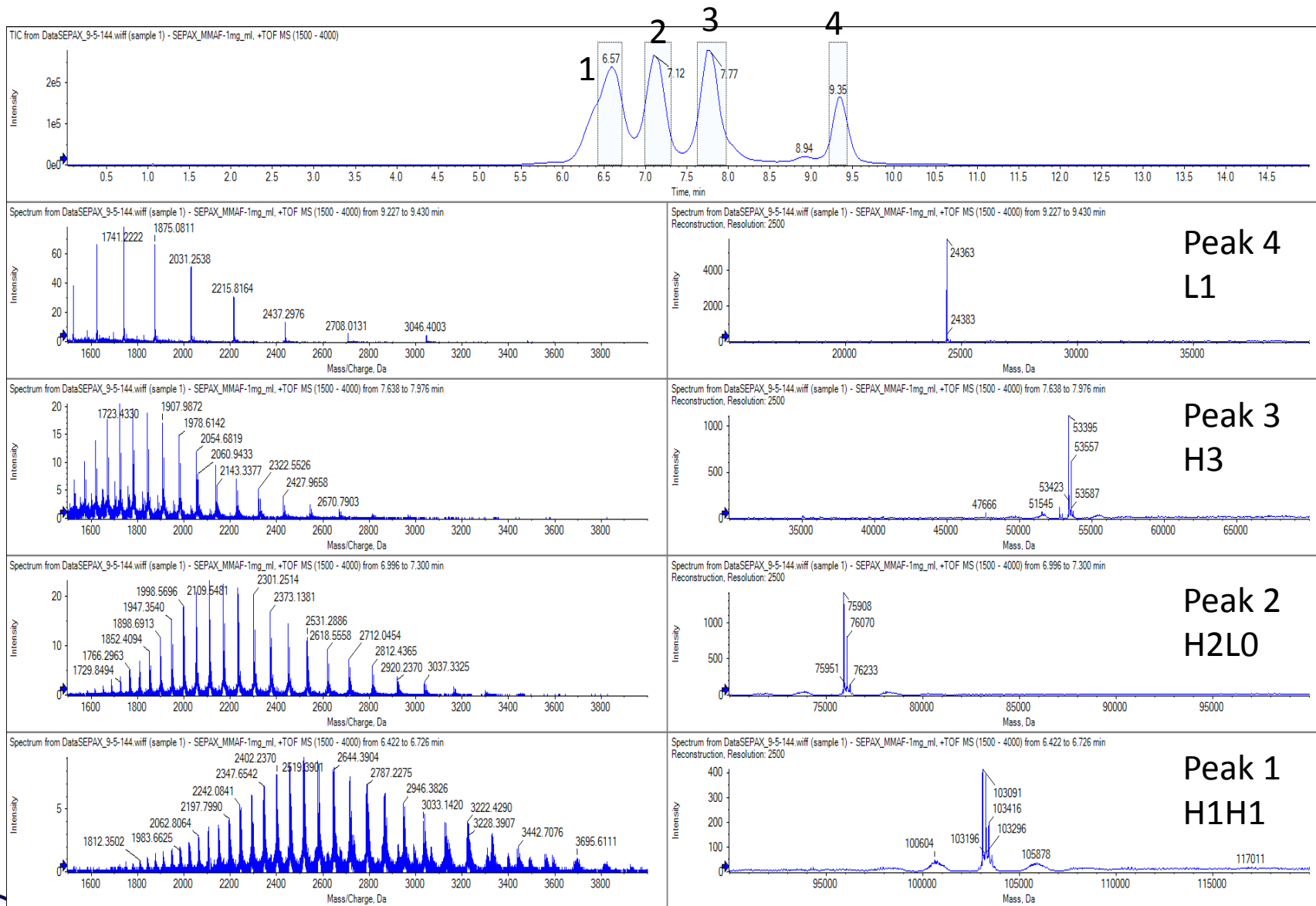


L1, H3

Non reduced: Intact 2, L1, H1HL, H2H1L, H1H1, LH2, H2H2, H3

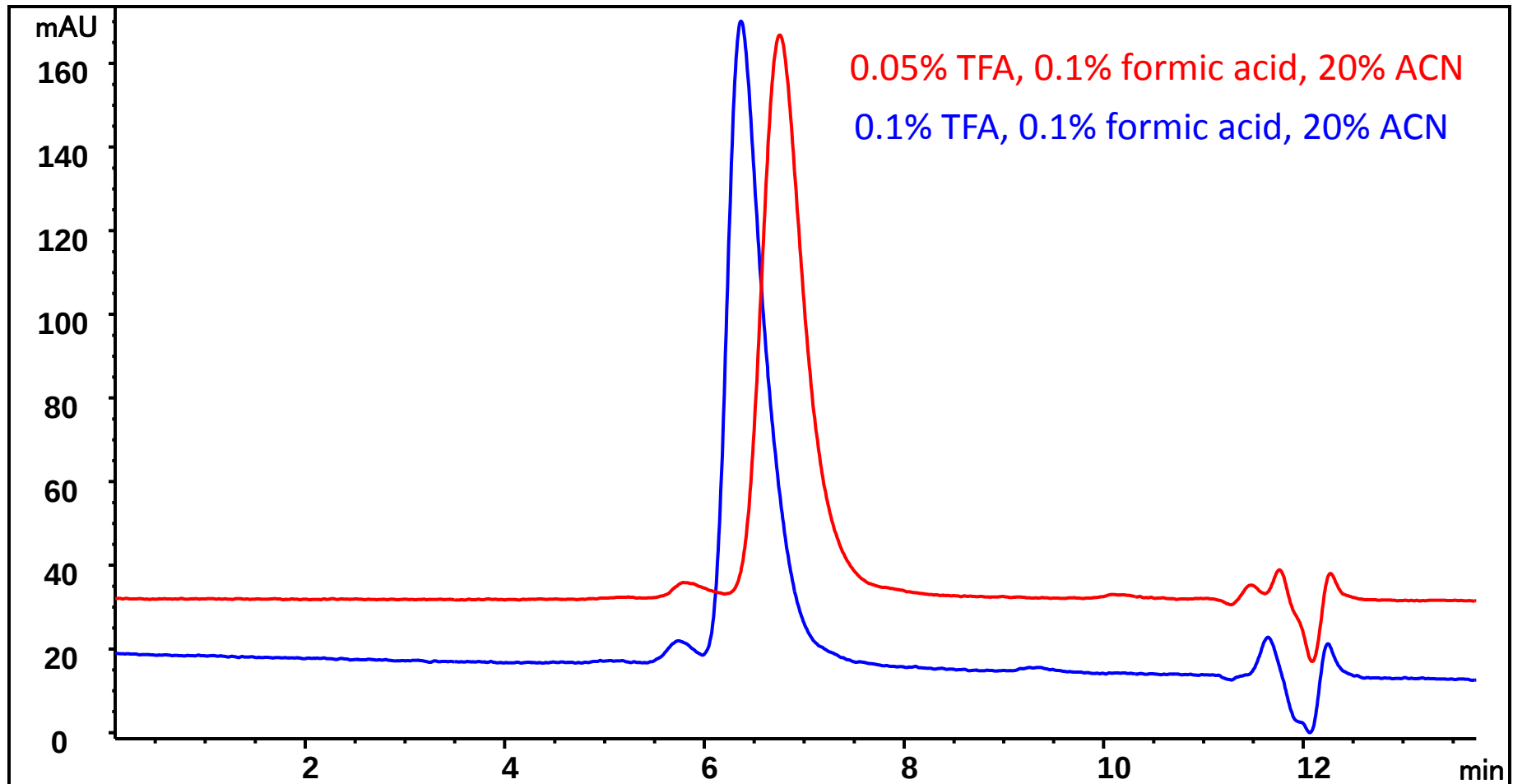
Fully reduced: L0, H0, L1, H1, H2, H3

Cysteine ADC SEC-LC/MS identification



TFA effect on the intact lysine ADC SEC separation

Column: Zenix-C SEC-300 (3 μm , 300 $\text{\AA}$, 4.6 x 300 mm), Mobile phase: 0.1% TFA, 0.1% formic acid and 20% ACN
Flow rate: 0.35 mL/min; Detector: UV 214 nm; Column temperature: 25 $^{\circ}\text{C}$
Injection volume: 2 μg , Sample: Herceptin ADCs 1mg/mL

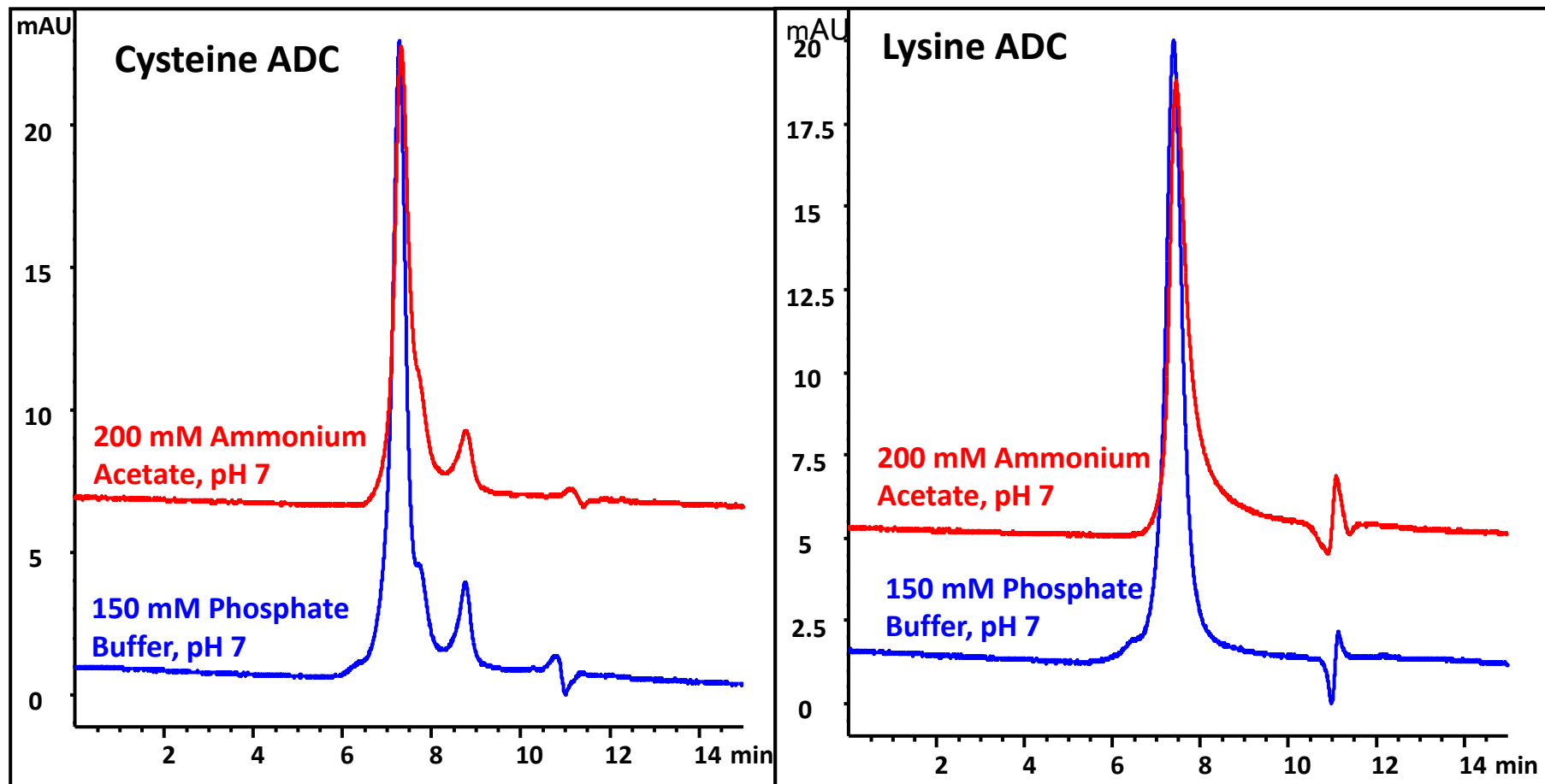


Intact Cysteine ADC and Lysine ADC separation under volatile native condition

Column: Zenix-C SEC-300 (3 μm , 300 $\text{\AA}$, 2.1 x 300 mm), Mobile phase: See chromatogram

Flow rate: 0.08 mL/min; Detector: UV 280 nm; Column temperature: 25 $^{\circ}\text{C}$

Injection volume: 1 μL , Sample: ADCs 1mg/mL



Product Information

Column	Part number
Zenix [®] SEC-300 (3 μm , 300 Å, 7.8 x 300 mm)	213300-7830
Zenix [®] SEC-300 (3 μm , 300 Å, 4.6 x 300 mm)	213300-4630
Zenix-C [®] SEC-300 (3 μm , 300 Å, 4.6 x 300 mm)	233300-4630
Zenix-C [®] SEC-300 (3 μm , 300 Å, 2.1 x 300 mm)	233300-2130