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Protein Quantitation using various Modes of High Performance Liquid Chromatography

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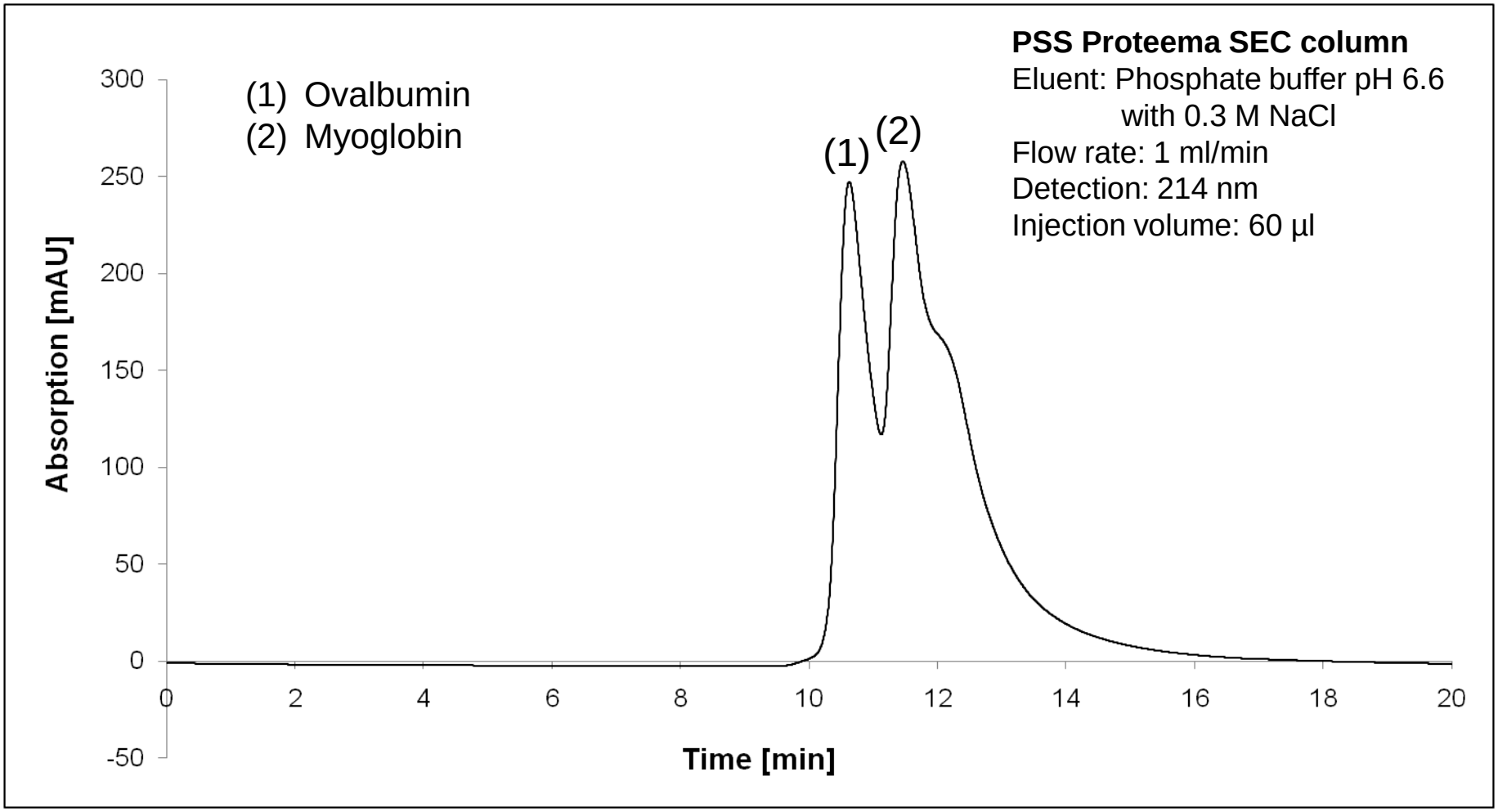
Innsbruck, Tuesday, September 20th

Quantitative Protein Analysis

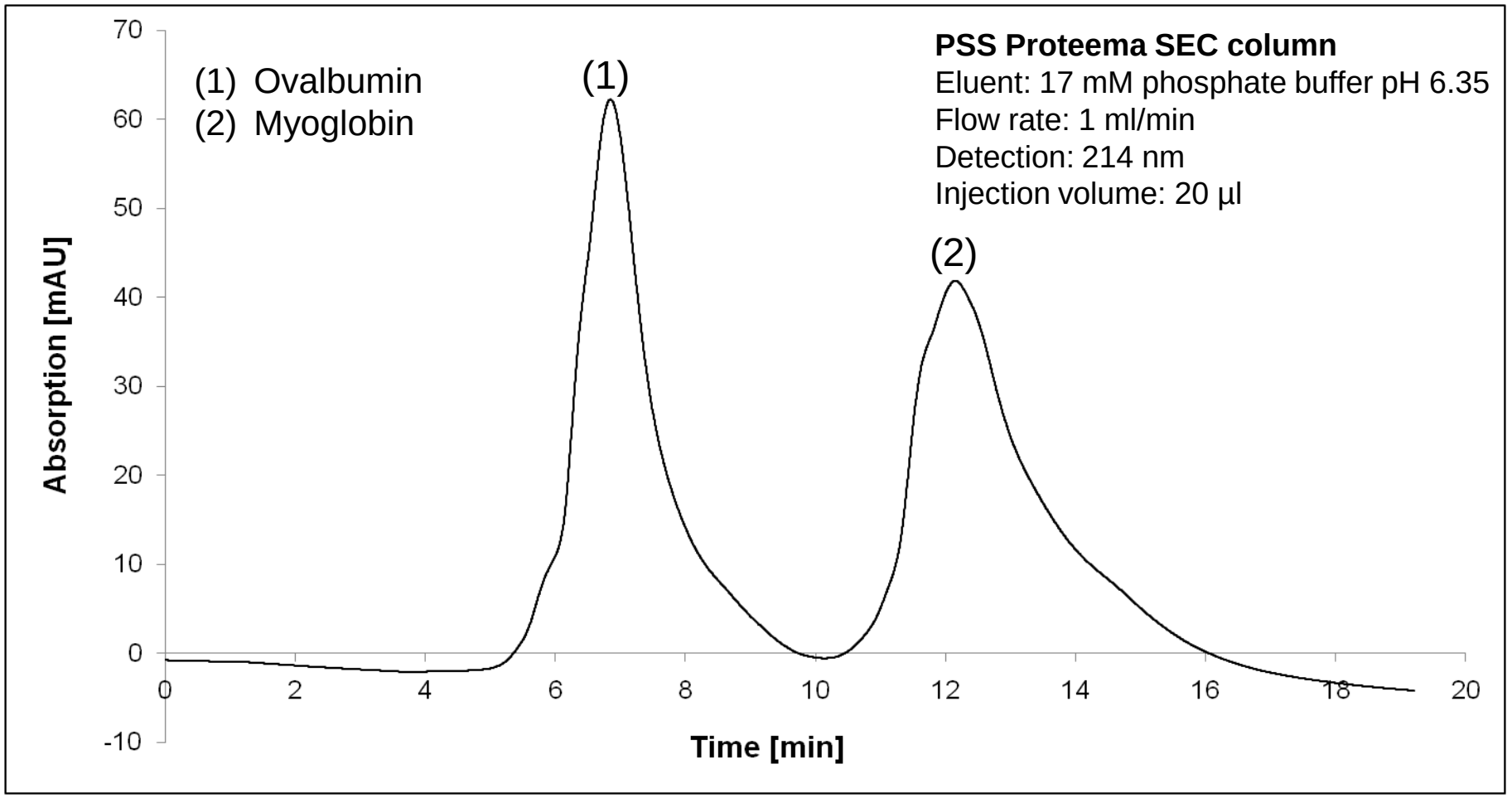
Protein Quantitation

Quantitative Protein Analysis by HPLC – Differences, similarities and evaluation

Ideal Size Exclusion Chromatography

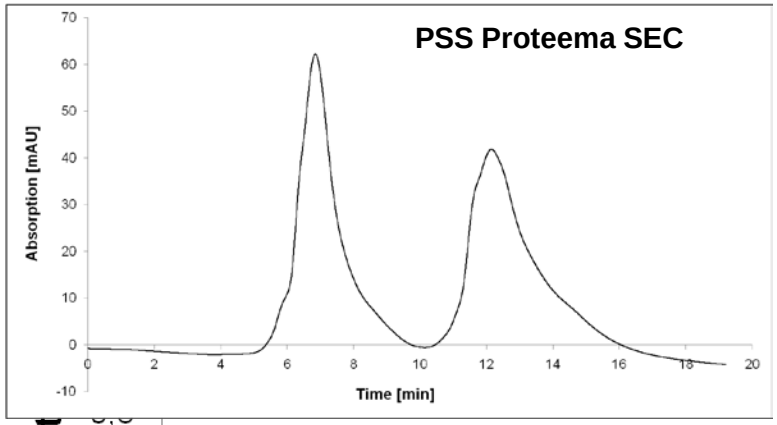


Non-Ideal Size Exclusion Chromatography



Concept according to: Kopaciewicz, W. and Regnier F.E., Anal. Biochem.;126 (1982) 8-16

Non-Ideal SEC - Phenomenex

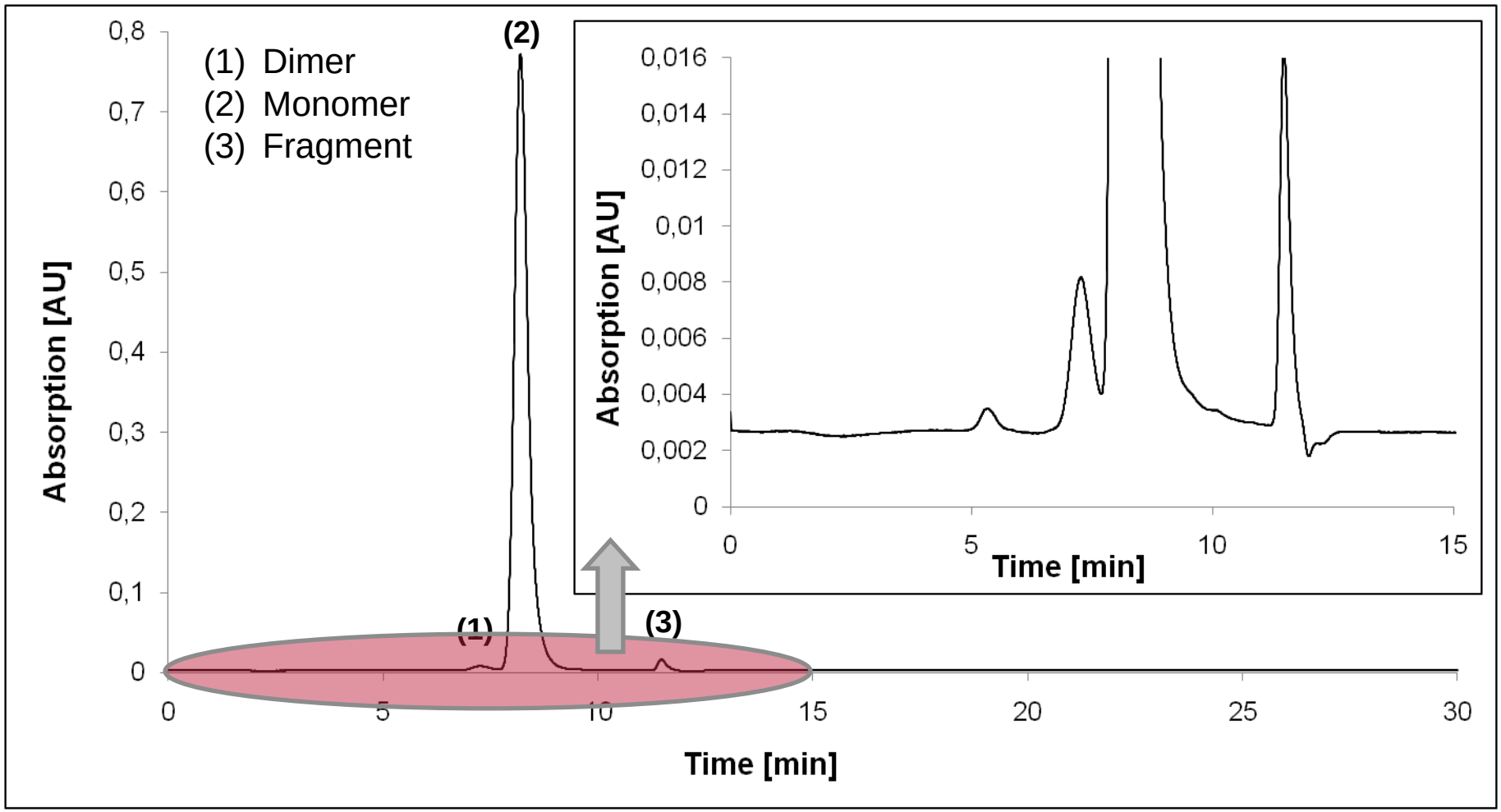


Phenomenex BioSep 3000
Eluent: 17 mM phosphate buffer pH 6.35
Flow rate: 1 ml/min
Detection: 214 nm
Injection volume: 40 µl

(1)
(2)

Absorpt
0,6
0,4
0,2
0
0 2 4 6 8 10 12 14 16 18 20
Time [min]

SEC of a monoclonal antibody



Method according to: Phenomenex BioSep Product manual, application: IgG aggregates

Routine analysis SEC data of mAb

Phast GmbH

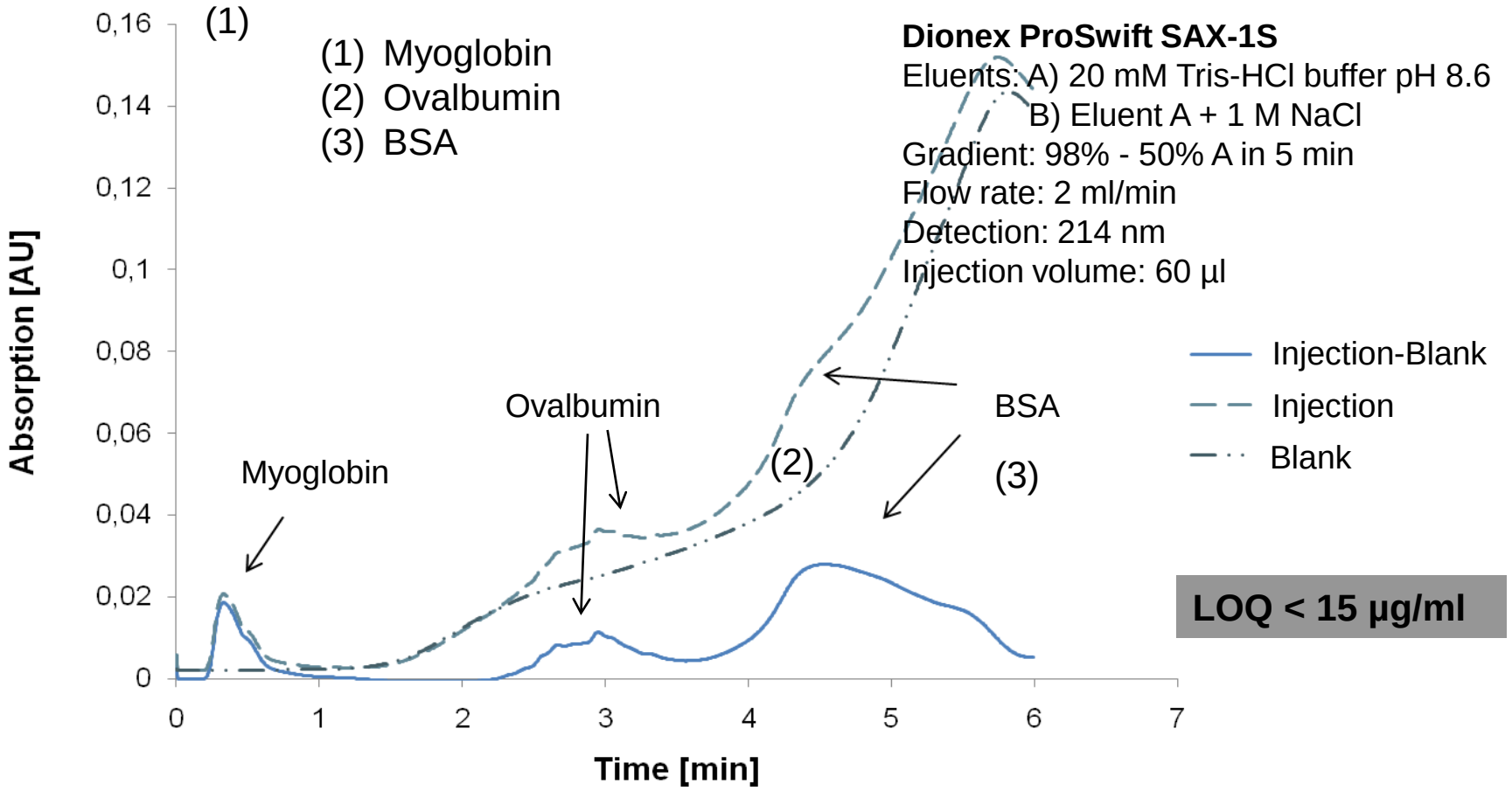
Sample	all Area
1	105900269
	105693289
	106003120
2	

A&M Stab

Sample	Area Monomer	Area Dimer
1	10329782	35288
	10339650	36192
	10334420	35415
		30813
		31602
		32405
		33411
		33618
		32709

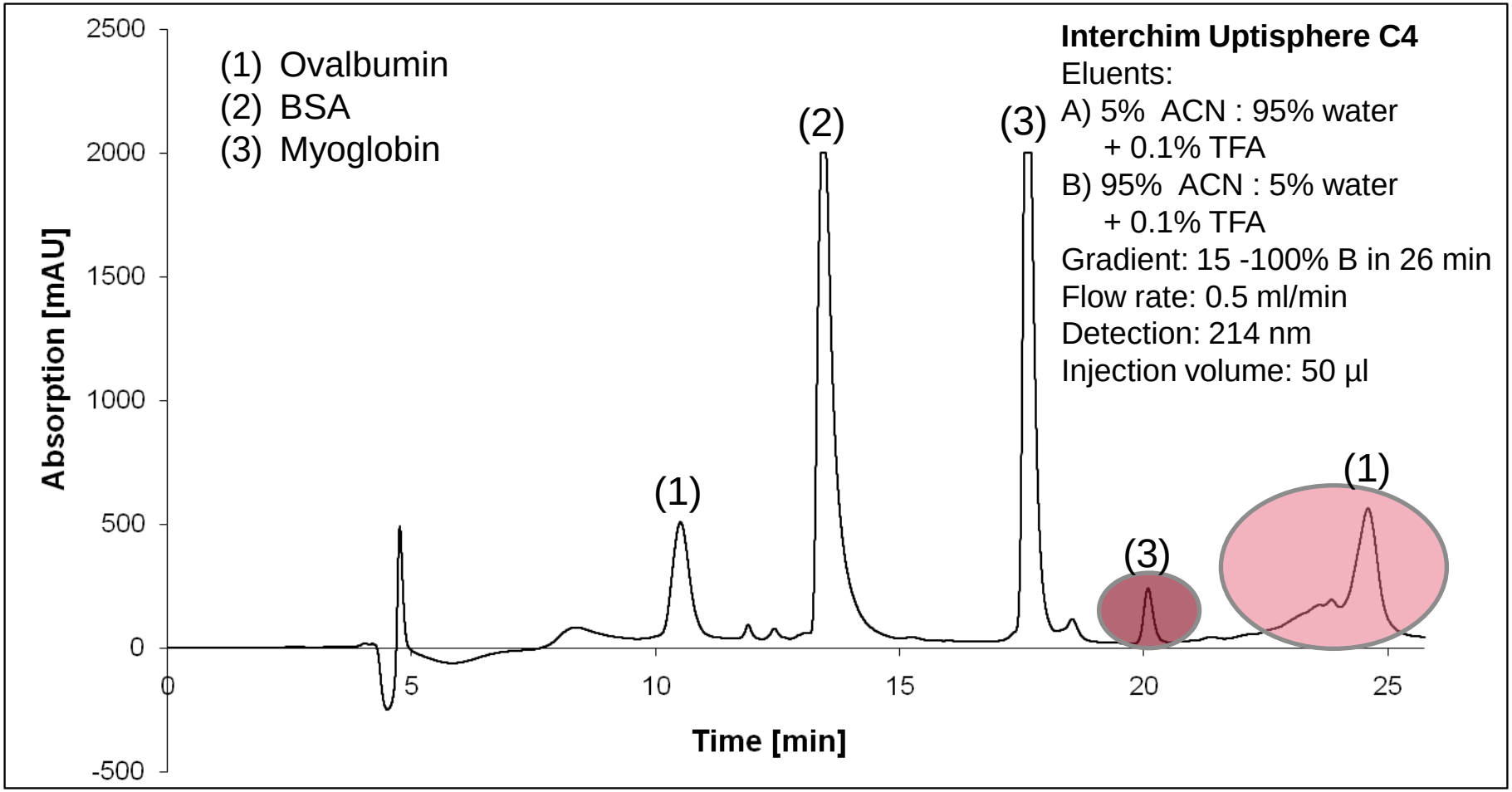
	RSD $\%_{pooled}$ area per sample	RSD $\%_{pooled}$ area per day (diff. samples)	RSD % area between days
Phast GmbH	0.373 (n=2)	0.452 (n=4)	1.86 (n=4)
A&M Stab • Monomer • Dimer	(n=3/5/6) 0.331 3.85	(n=9/15/18) 1.86 6.46	(n=4) 2.11 24.1

Strong Anion Exchange Chromatography



Method according to: Dionex Corp., Product manual for ProSwift monolithic columns (2008) 44-45, modified

RP-HPLC



Modified according to: Bradshaw, T.P., Phenomenex; Introduction to Protein and Peptide HPLC (1998) 31-32

Routine analysis - external RP data (Phast GmbH)

Detection: DAD

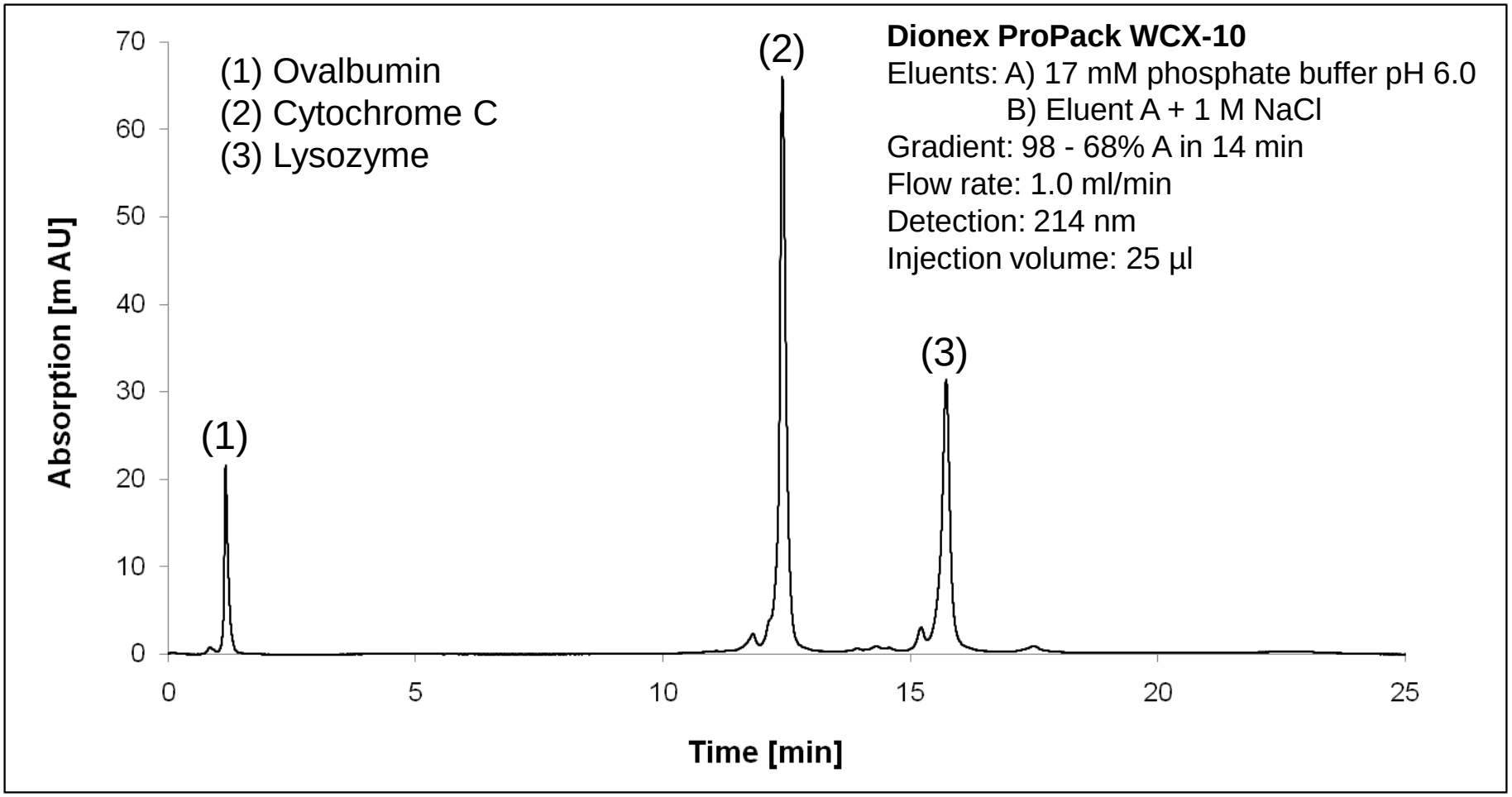
Sample	Area
1	7756503
	7994933
	7948043
	7963330
2	7956266

Detection: FLD

Sample	Area 1	Area 2
	169652834	105984746
	169639282	105862960
		87996
		66062
		45881
		01395
		76901
		35946
		15025

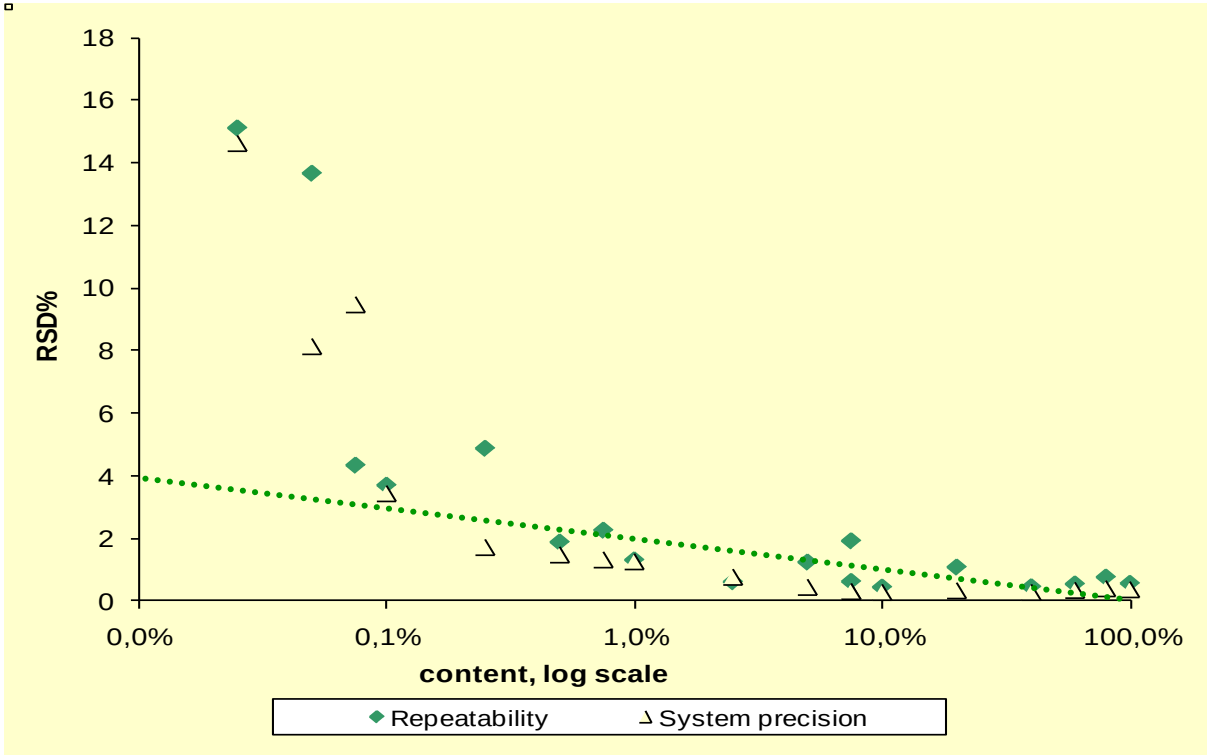
Method/ Detection	RSD $\%_{pooled}$ area per sample	RSD $\%_{pooled}$ area per day (dif. samples)	RSD % area between days
DAD	1.69 (n=3)	1.47 (n=9)	5.76 (n=6)
FLD • Peak 1 • Peak 2	(n=5/2) 0.347 0.390	(n=9) 1.26 1.41	(n=7) 3.81 2.65

Weak Cation Exchange Chromatography



Method according to: Dionex Corp., Product manual for ProPac IEX columns (2007) 29-30

Quantitative Protein Analysis by HPLC: Similarities



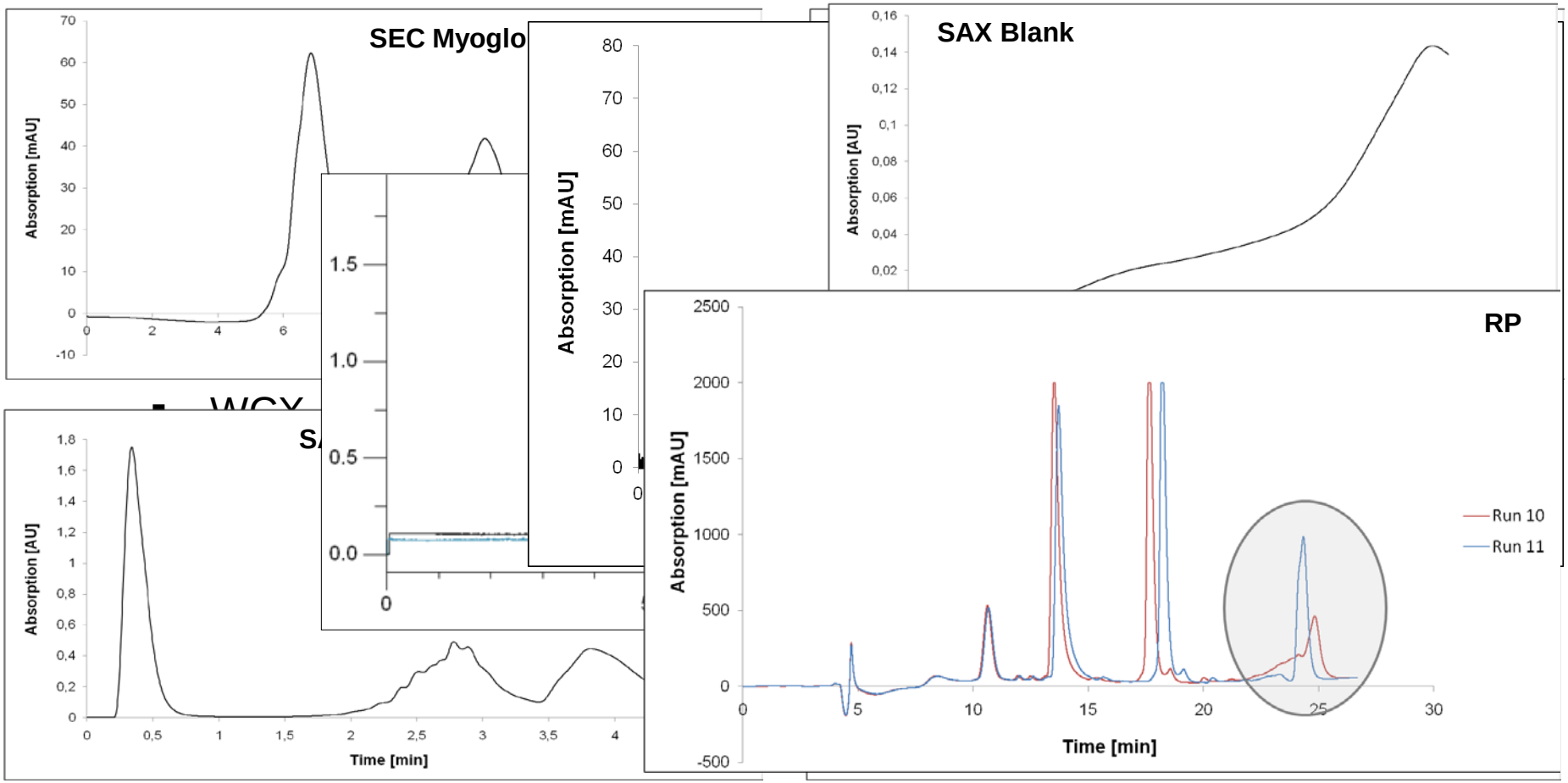
- Concentration dependence

  Udo Schepers, Joachim Ermer, Lutz Preu, Hermann Wätzig

  J. Chromatogr. B, 810, 111-118 (2004)

Quantitative Protein Analysis by HPLC: Differences

- Separation mechanisms, selectivity



Overall Conclusions

	Peak capacity [peaks/10 min] best	Peak capacity [peaks/10 min] worst	Selectivity	Precision	Analysis time [min]	LOQ [µg/ml]	Sample/ Mobile phase preparation
SEC • PSS • Phe	2.22 6.67	1.67 5.00	-	++	16	< 4	+++
SAX	13.3	6.67	+	+	5 (+20)	< 15	+
WCX	20.0	8.33	+	+	17 (+18)	< 10	+
RP	16.7	5.00	+(+)	++	26 (+22)	< 15	++
SEC mAb	14.5	4.85	+	+(+)	12	not yet measured	+++

WCX	3.25		1.67	2.10	3.46		1.90	2.09
	Dimer	**Monomer**	**Fragment**	**all area**	**Dimer**	**Monomer**	**Fragment**	**all area**
mAb	4.31	0.663	3.02	0.644	6.46	2.54	22.7	1.95

CE data: Suratman, A. , Wätzig, H.; J. Sep. Sci., 2008, 31, 1834-40 and unpublished

Further possible improvements

- Method development for analysis of monoclonal antibodies with WCX, RP-4 and SAX

  ⇨ Strategies for individual method optimization

- Analysis of Variance with data from protein analysis

  ⇨ Role of sample pretreatment

Acknowledgements

- interchim, Montlucon (Fra)
- PHAST quality standards, Homburg (Ger)

  Markus Limberger, Andreas Ziegler
- A&M Labor für Analytik, Mainz (Ger)

  Steven Watt

Chromatographic conditions

	SEC non ideal	SAX	WCX	RP
mobile Phase Eluent A	phosphate buffer	tris-HCl buffer	phosphate buffer	5% ACN : 95% water + 0.1% TFA
Eluent B	Ø	Eluent A + 1 M NaCl	Eluent A + 1 M NaCl	95% ACN : 5% water +0.1% TFA
gradient	Ø	linear 98% - 50% A	linear 98%-68% A	linear 15%-100% B
ionic strength	17-18 mM	20 mM	17-18 mM	Ø
pH-value	6.35	8.6	6.0	~ 1.9
flow rate	1 ml/min	2 ml/min	1 ml/min	0.5 ml/min
analysis time	16 min	5 min	17 min	26 min
injection volume	40 µl	60 µl	25 µl	50 µl
detection wavelength	214 nm	214 nm	214 nm	214 nm

Chromatographic conditions - Matuzumab

	SEC	RP
mobile Phase Eluent A	phosphate buffer	5% ACN : 95% water + 0.1% TFA
Eluent B	Ø	95% ACN : 5% water +0.1% TFA
gradient	Ø	isocratic 50% A : 50% B
ionic strength	100 mM	Ø
pH-value	6.80	~ 1.9
flow rate	1 ml/min	0.5 ml/min
analysis time	15 min	12 min
sample content	2.5 mg/ml	3.3 mg/ml
injection volume	10 µl	50 µl
detection wavelength	214 nm	214 nm

Protein properties

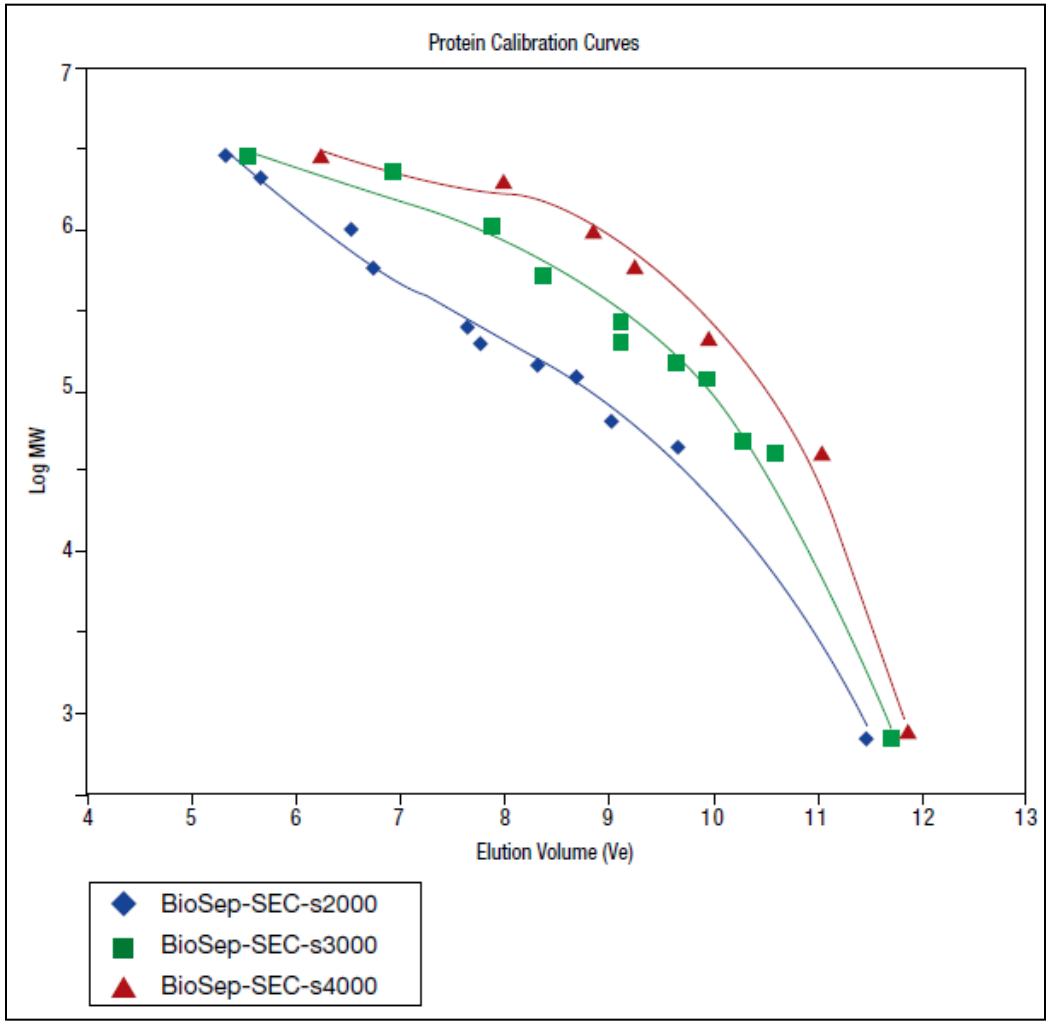
Proteins	MW [Da]	pI
Myoglobin	17,000	6.99
Ovalbumin	45,000	4.9
BSA	66,000	4.2-4.9
Cytochrome C	13,000	10.1
Lysozyme	14,300	11.1

Dumetz, A.C. et al, Protein Scie.; 16 (2007) 1867-1877
Gochman-Hecht, H., Bianco-Peled, H.; J. Coll. Interf. Scie. 297 (2006) 276-283

HPLC systems

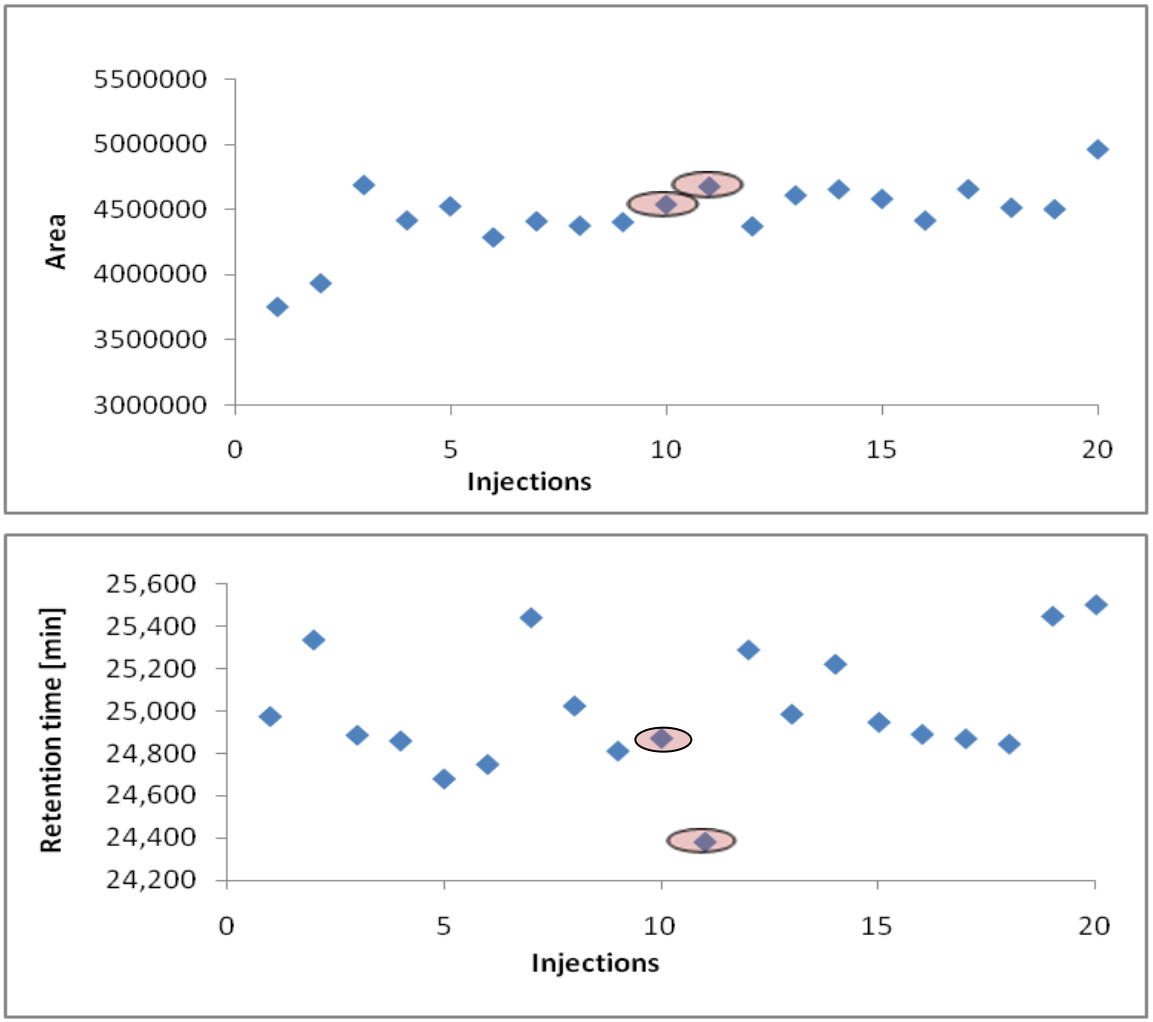
	SAX	SEC/RP/WCX
	Hitachi Merck	Elite LaChrom (VWR)
Solvent pump	L-6200A	L-2130
Autosampler	AS-2000A	AS 2203
Detector (UV-Vis)	L-4250	L-2400
Interface	D-6000	organizer
Software	D7000 HSM (Merck)	EZ Chrom Elite Vers. 3.2.1

Size determination of Matuzumab

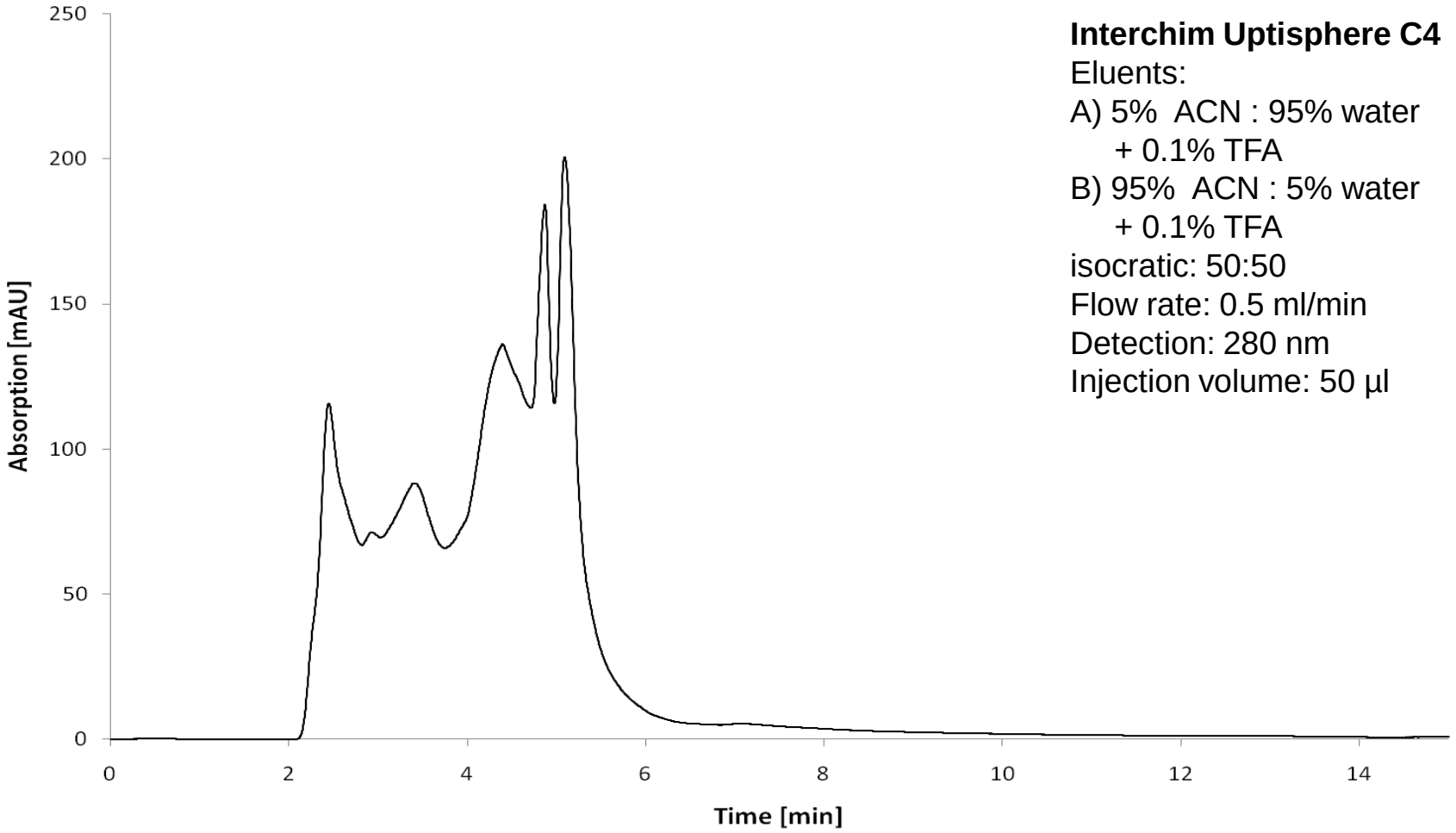


Phenomenex BioSep Product manual; calibration curve

Control charts RP – peak shapes



RP-Separation of Matuzumab (EMD 72000) + DTT



Interchim Uptisphere C4
Eluents:
A) 5% ACN : 95% water
+ 0.1% TFA
B) 95% ACN : 5% water
+ 0.1% TFA
isocratic: 50:50
Flow rate: 0.5 ml/min
Detection: 280 nm
Injection volume: 50 µl

SEC of an IgG antibody + DTT