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Ultra-sensitive simultaneous LC-MS/MS quantification of human insulin, glargine, lispro, aspart, detemir and glulisine in human plasma using 2D-LC and a novel high efficiency column

Erin E. Chambers
Principal Applications Chemist

Outline

- Background and Goals
- Mass spectrometry development
- Chromatography development
- Sample Preparation Development
- Validation Data
- Conclusions

Insulin and Analogs

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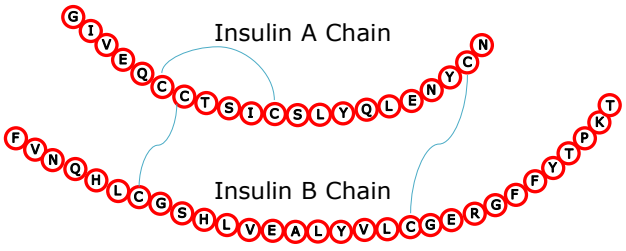
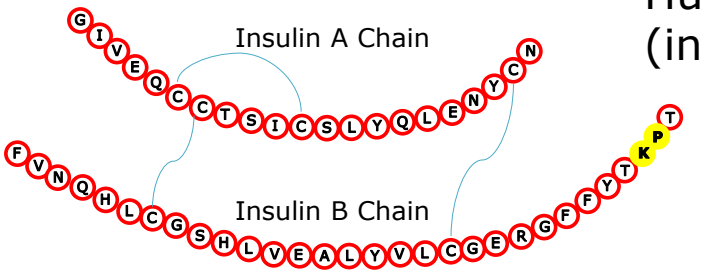
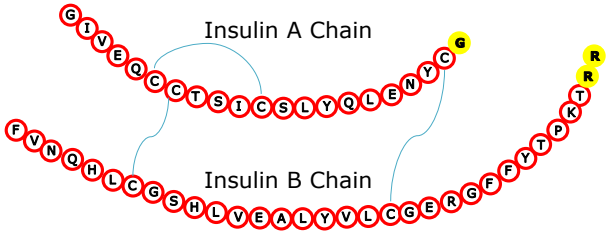
Human Insulin
MW 5808



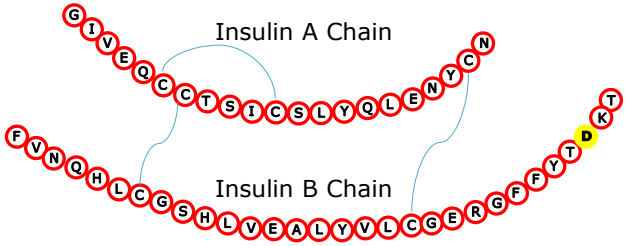
Humalog
(insulin lispro)



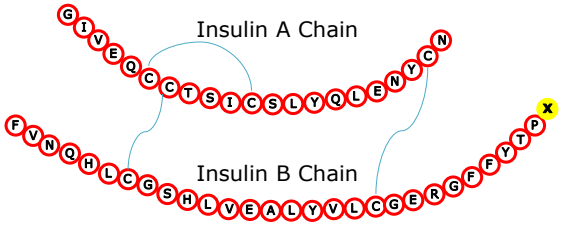
Insulin glargine
(Lantus®)
Avg MW 6063



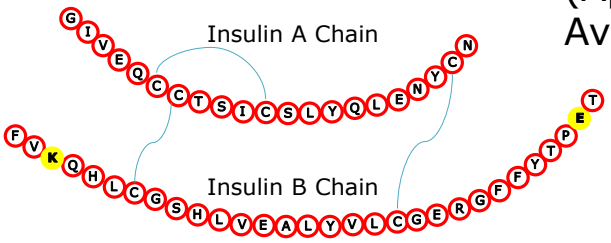
Insulin aspart
(Novalog®)
Avg MW 5826



Insulin detemir
(Levemir®)
Avg MW 5917

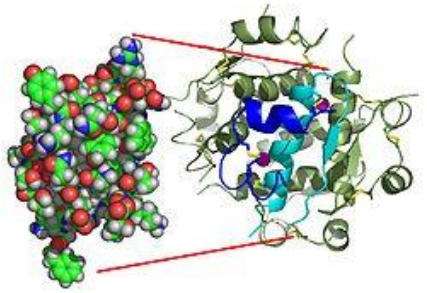


Insulin glulisine
(Apidra®)
Avg MW 5823



Background

Why bioanalysis for insulin analogs?



1. Many coming off patent between 2013 and 2015
   - Bioequivalence studies
   - Development of new versions
     - bioanalysis
2. Methods needed to identify/differentiate *specific* insulins
   - Need simultaneous quantification as combination therapies common
   - Forensic toxicology, cases of wrongful death
   - Anti-doping
   - Understanding/monitoring of patient dosing?

- Current analytical methods
  1. ELISA- based assays (lack of standardization)
  2. Nano-flow or low flow LC-MS/MS assays
  3. SPE-immuno affinity LC-MS/MS assays
  4. Assays where insulin has been digested or disulfide bonds reduced

Specific Challenges in Developing an LC-MS/MS Assay for Insulin Analogs

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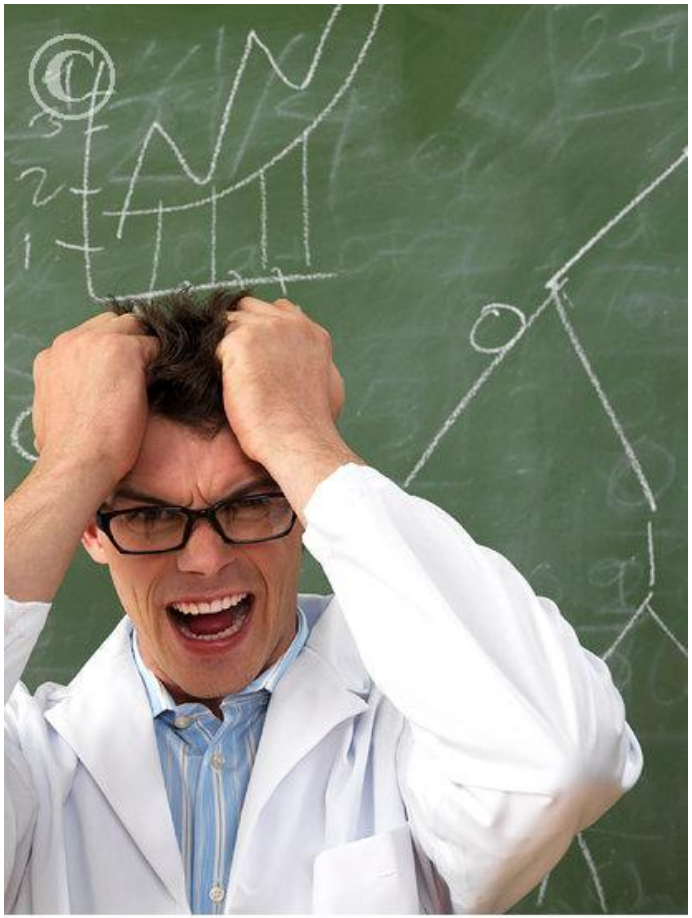
- Key challenge: distinguish human insulin and Humalog (lispro) whilst obtaining adequate specificity for low level detection
- Obtain sensitivity similar to LBAs
  - Specificity in matrix
  - High level of non-specific binding (NSB)
  - Low MS sensitivity
    - Poor fragmentation
    - Multiple precursors
  - Chromatographic peak shape
  - Protein binding



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MS scan of Insulin Glargine

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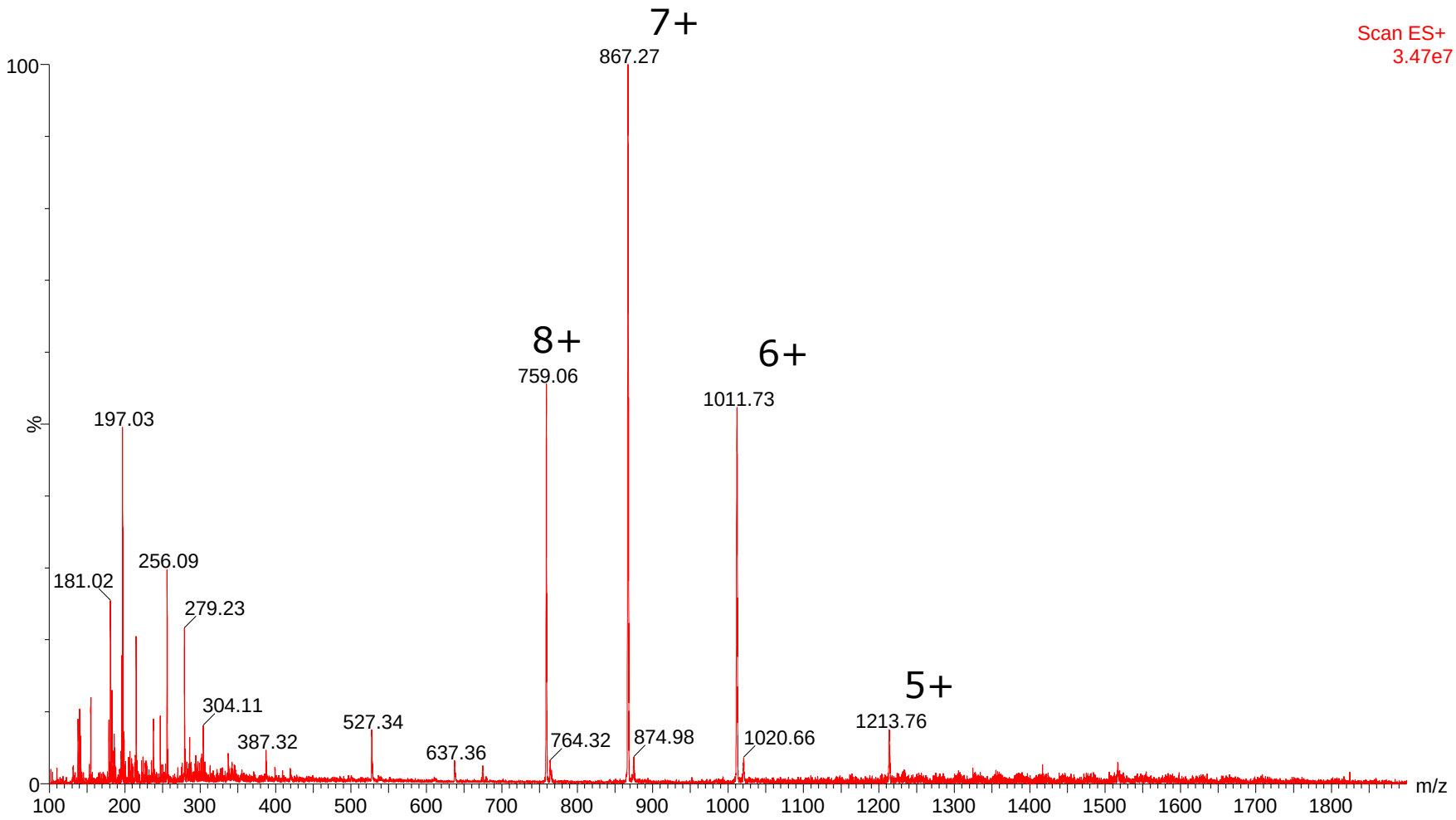

Lantus infused at 10 µL/min teed into LC effluent containing 40% ACN

MS Specificity: Avoiding Immonium Ion Fragments

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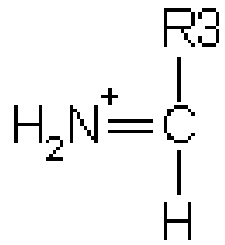
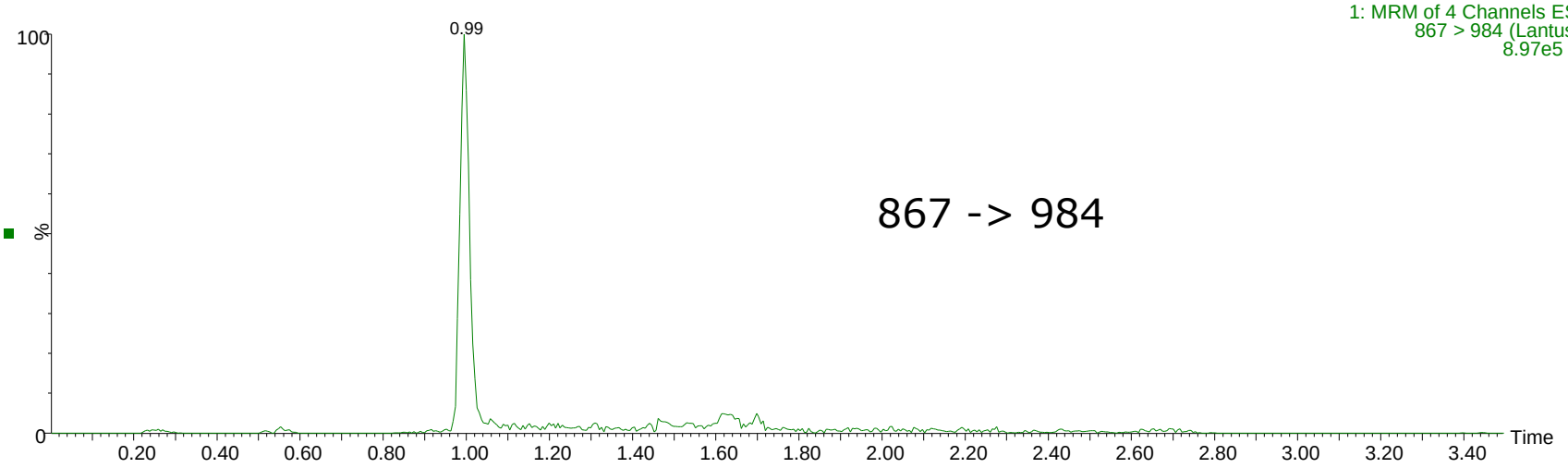


867 -> 136 (tyrosine immonium ion)

Lack of Specificity



867 -> 984

MSMS spectra for insulin glulisine, aspart, detemir, and glargine

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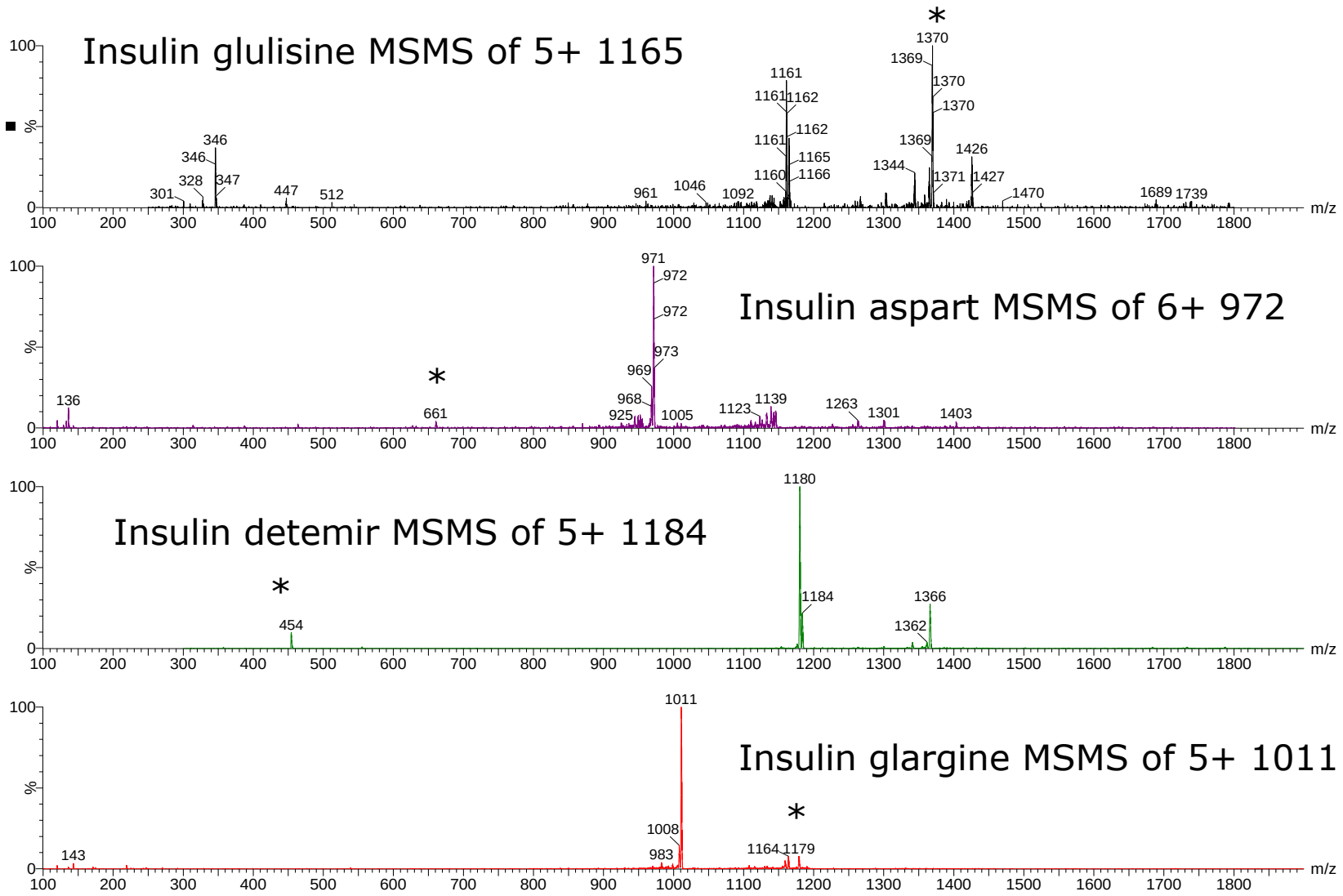

MSMS spectra from 5+ precursors of human insulin and insulin lispro

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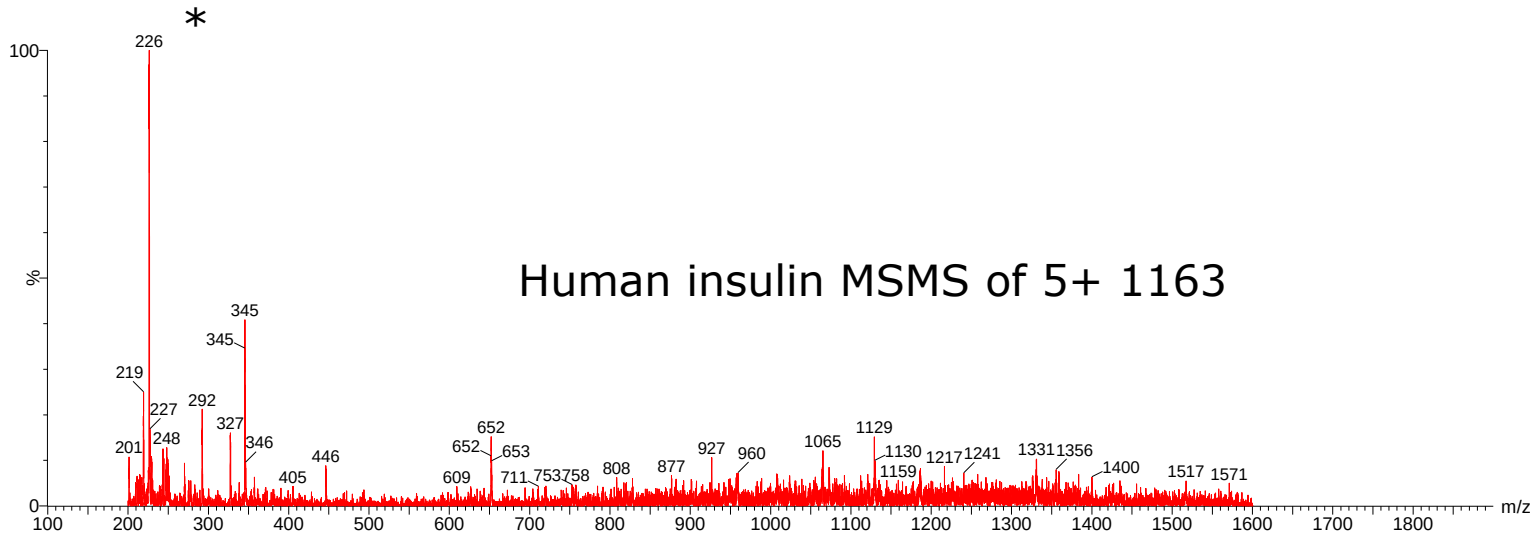

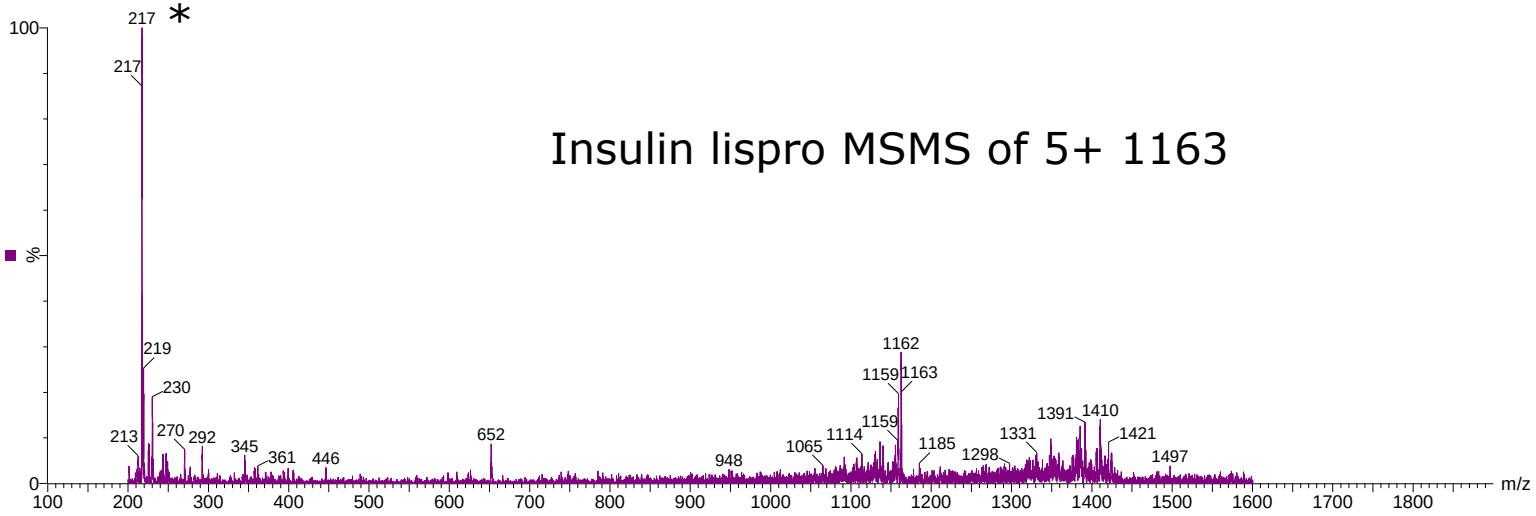

MSMS of different Humalog (lispro) Precursors

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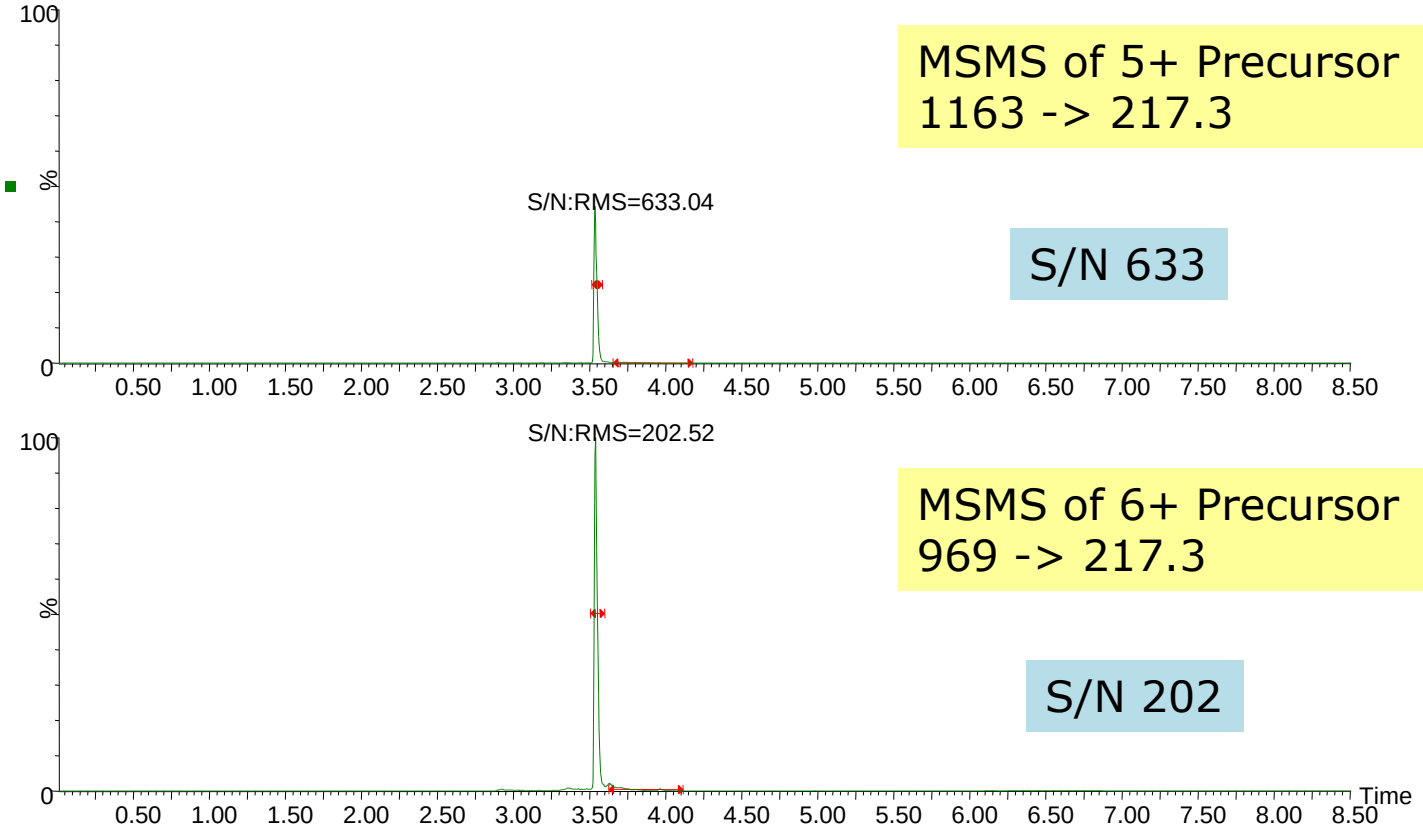
Lower m/z precursor yields higher intensity but lower signal to noise



MSMS of 5+ Precursor
1163 -> 217.3

S/N 633

MSMS of 6+ Precursor
969 -> 217.3

S/N 202

Humalog Sample Analysis: Effect of Higher m/z Precursor

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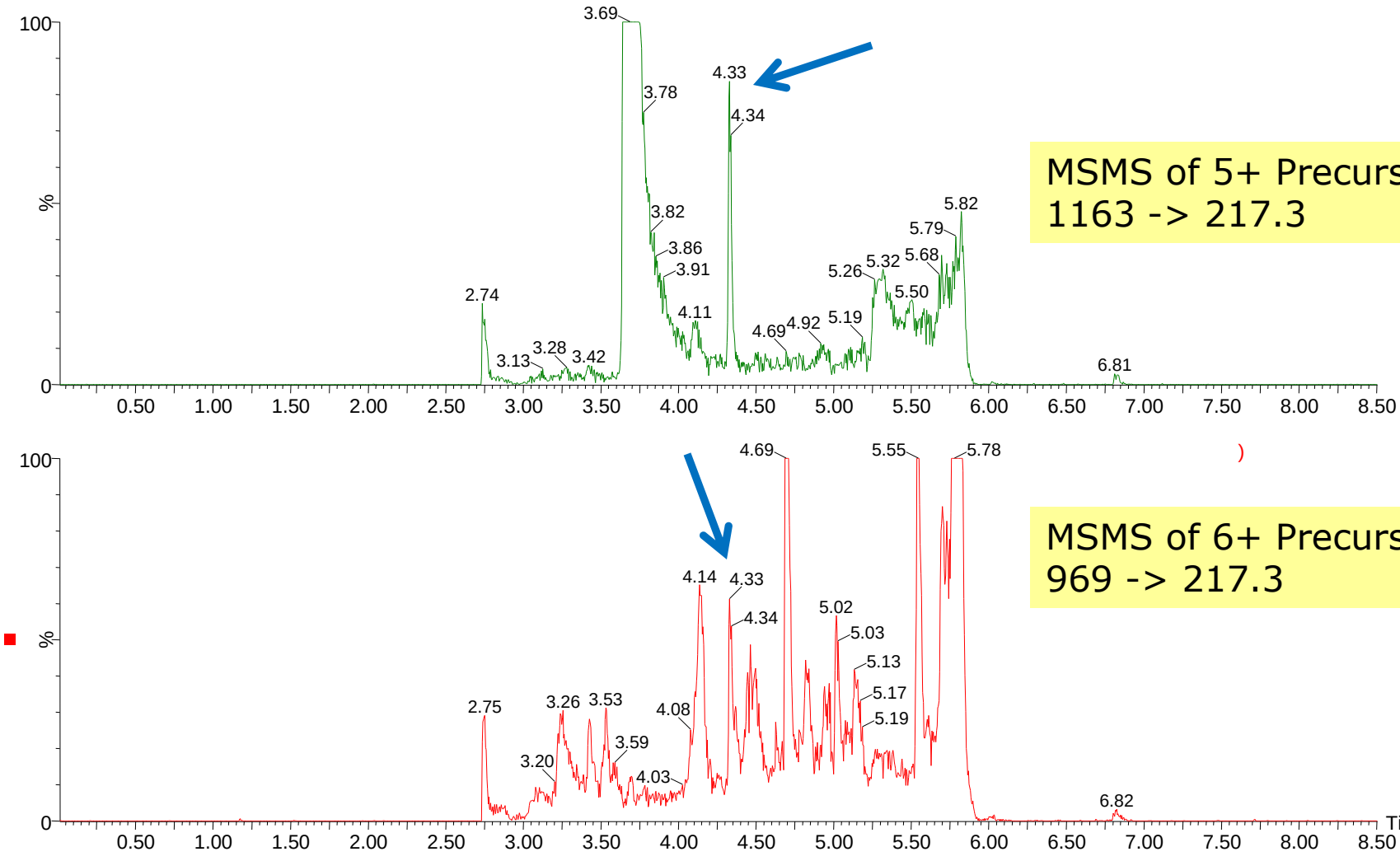

Xevo TQ-S Triple Quadrupole MS conditions

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Specific Insulin	MRM Transition	Cone Voltage (V)	Collision Energy (eV)
Glargine	1011->1179	60	25
	867->984	60	18
Lispro	1162-> 217	50	40
	968.5->217	50	40
Detemir	1184-> 454.4	60	20
	1184-> 1366.3	60	20
Aspart	971.8 -> 660.8	60	18
	971.8 -> 1139.4	12	18
Glulisine	1165 -> 1370	14	22
	1165 -> 346.2	14	22
Bovine (IS)	956.6 -> 1121.2	60	18
Human insulin	1162 -> 226	50	40
	968.5->217	50	40

Note: highlighting indicates the primary transitions used for quantification

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Evolution of Insulin Method: Traditional C18 to Charged Surface Hybrid (CSH™) C18

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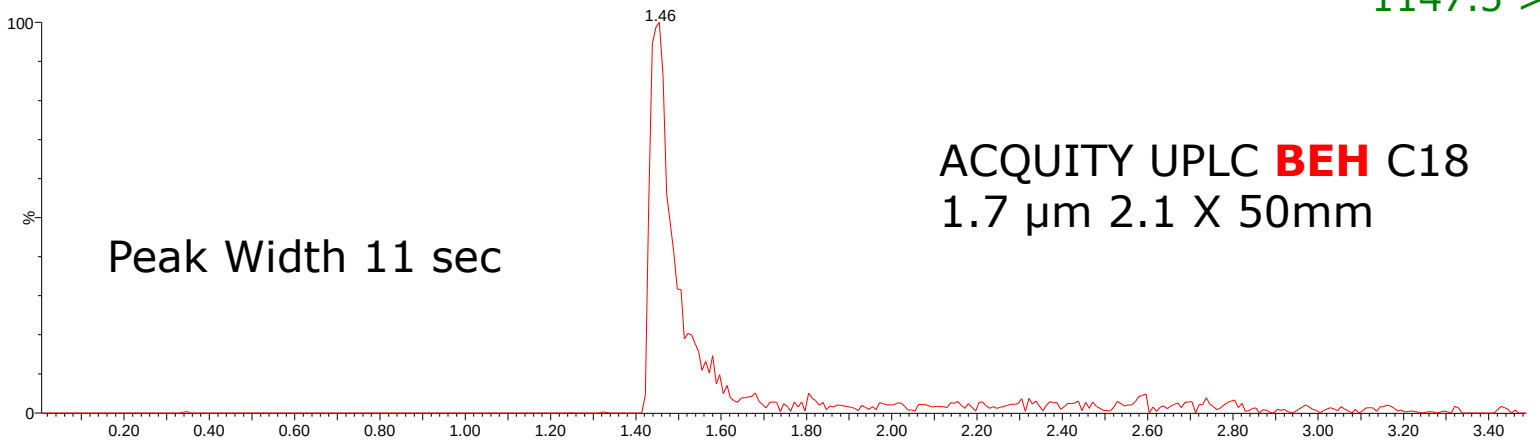
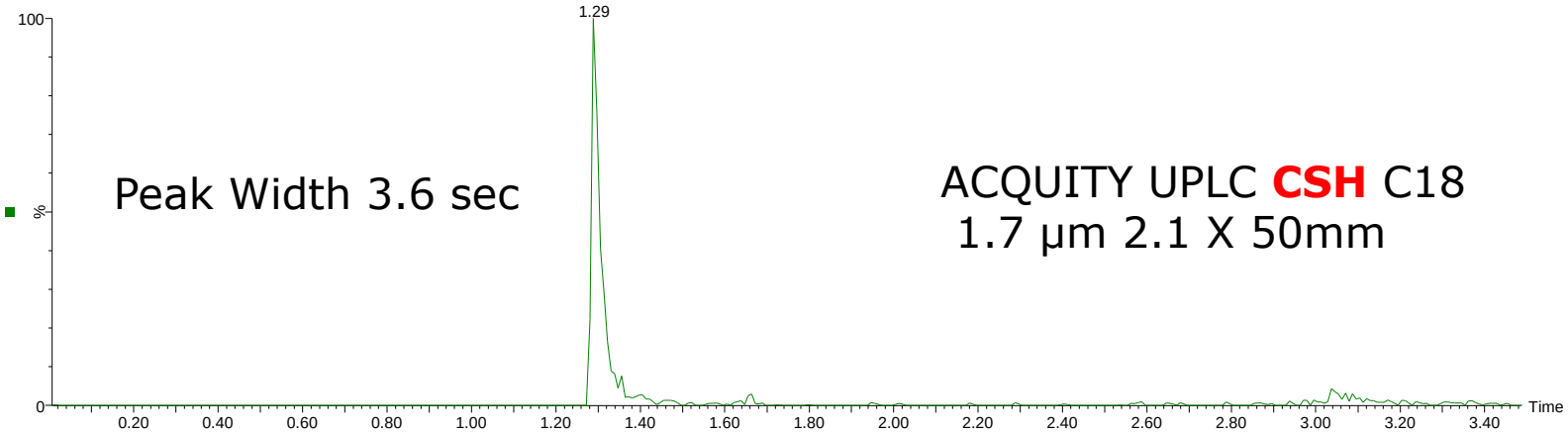
Bovine Insulin MW 5734

1147.5 > 315.2



Peak Width 11 sec

ACQUITY UPLC **BEH** C18
1.7 µm 2.1 X 50mm



Peak Width 3.6 sec

ACQUITY UPLC **CSH** C18
1.7 µm 2.1 X 50mm

Improvement for insulin using solid core charged surface columns

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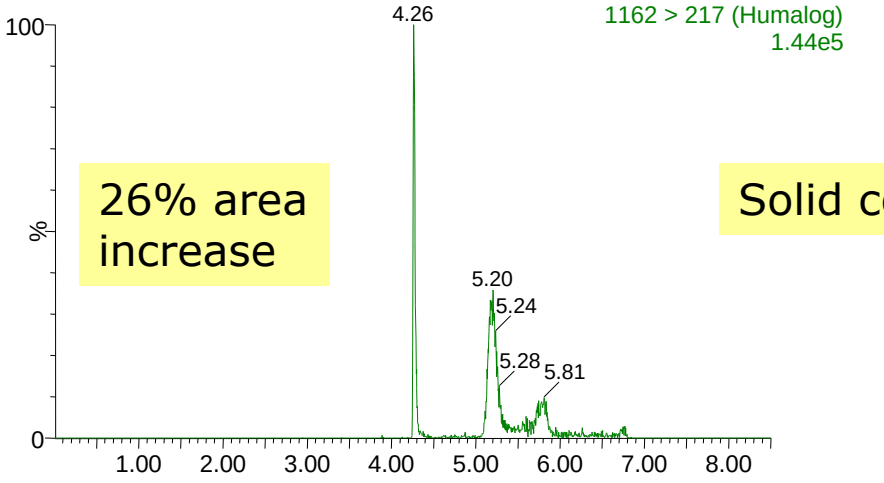
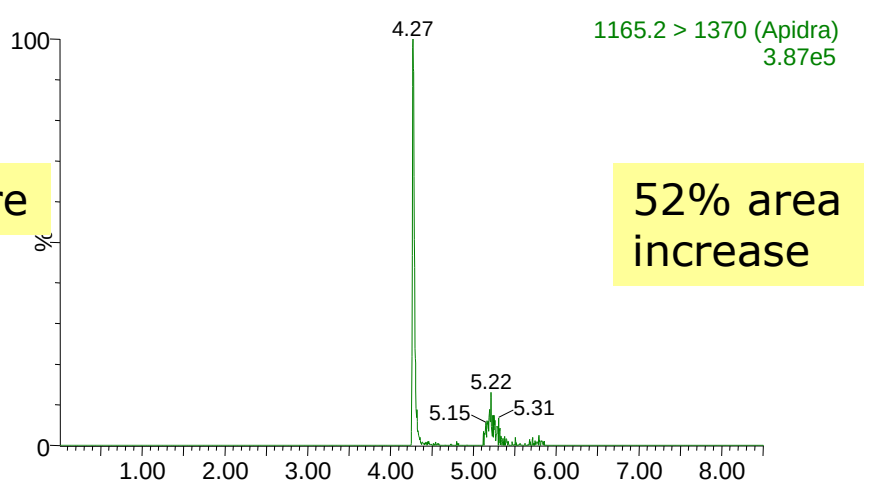
Humalog

Apidra

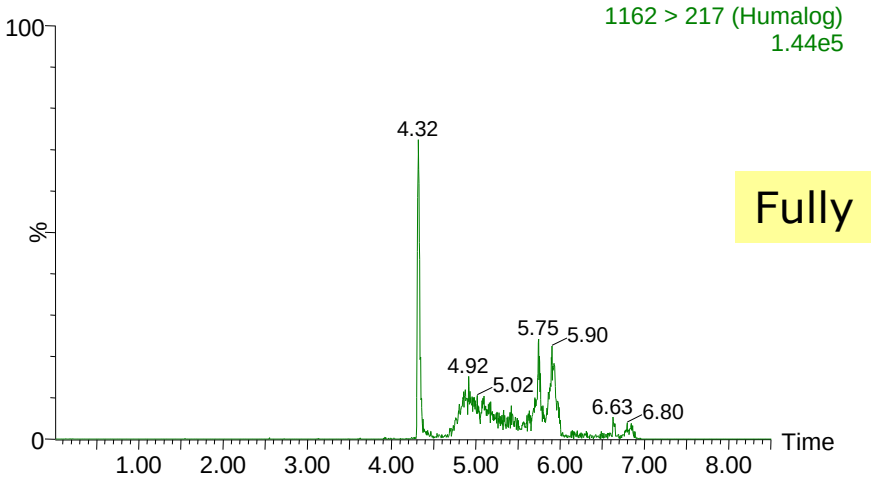


26% area increase

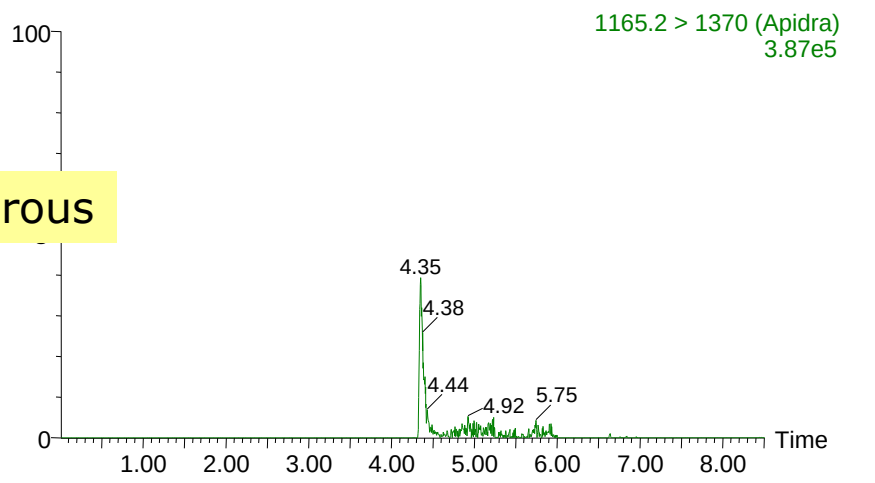
Solid core



52% area increase



Fully porous



LC Method: At-column-dilution and Trap and Back Elute

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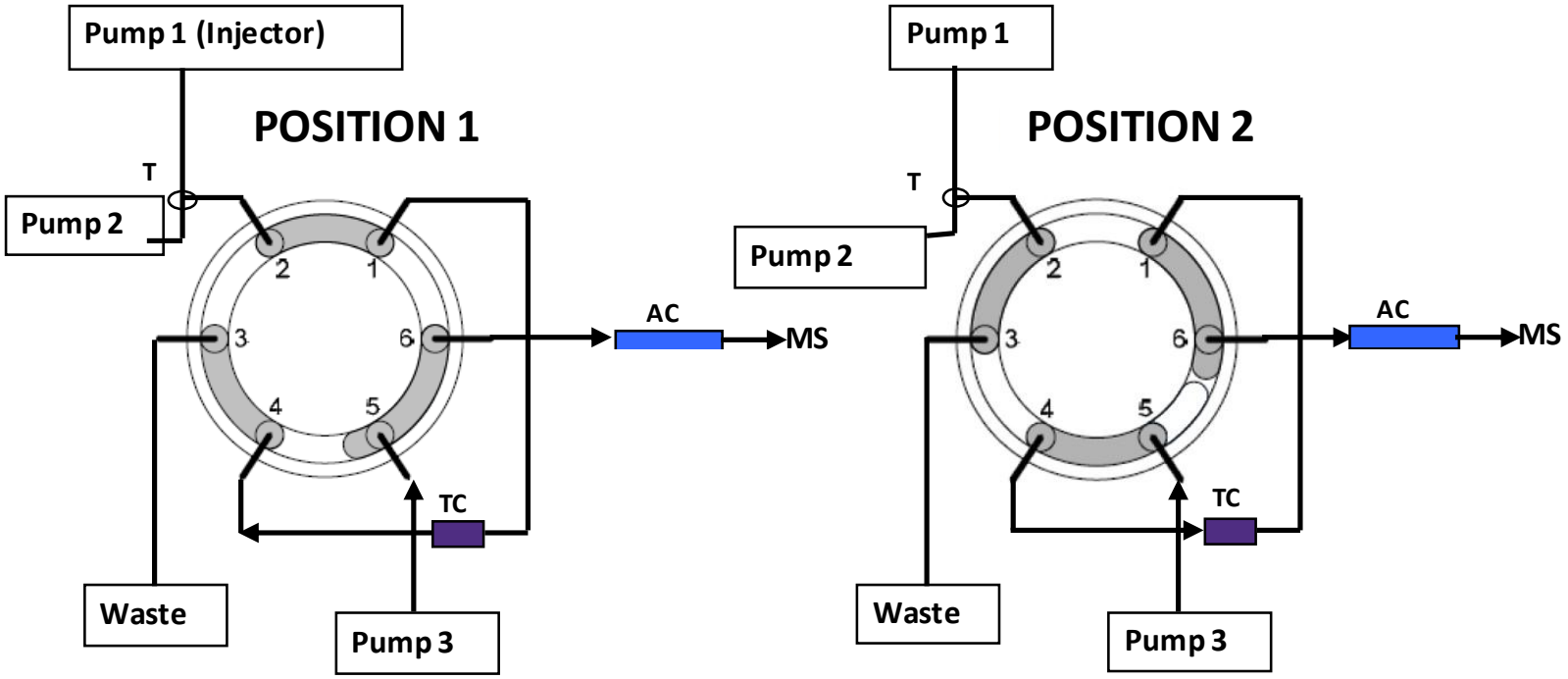
- Analytical Column: CORTECS C18+ 2.1 X 50mm, 1.7 µm
- Trap column: XBridge C18 IS, 3.5 µm, 2.1 X 20mm
- Mobile phase A= 0.1% formic acid in water
- Mobile phase B= 0.1% formic acid in ACN
- Loading time: 2 minutes
- At Column Dilution
- Elution
  - 15 to 40% B over 4 minutes
- Analytical Column Temp: 60° C
- Sample Temp: 15° C
- Injection Volume: 30 µL (can inject 45 µL without breakthrough)
- SNW: 50/25/24/1 ACN/IPA/H20/FA

ACQUITY IClass with 2D Technology: Valve Diagram



Pump 1: Loading pump
Pump 2: Dilution pump
Pump 3: Elution pump

TC= trapping column
AC= analytical column

UPLC-MS/MS Chromatograms of human insulin, insulin analogs, and bovine insulin (IS)

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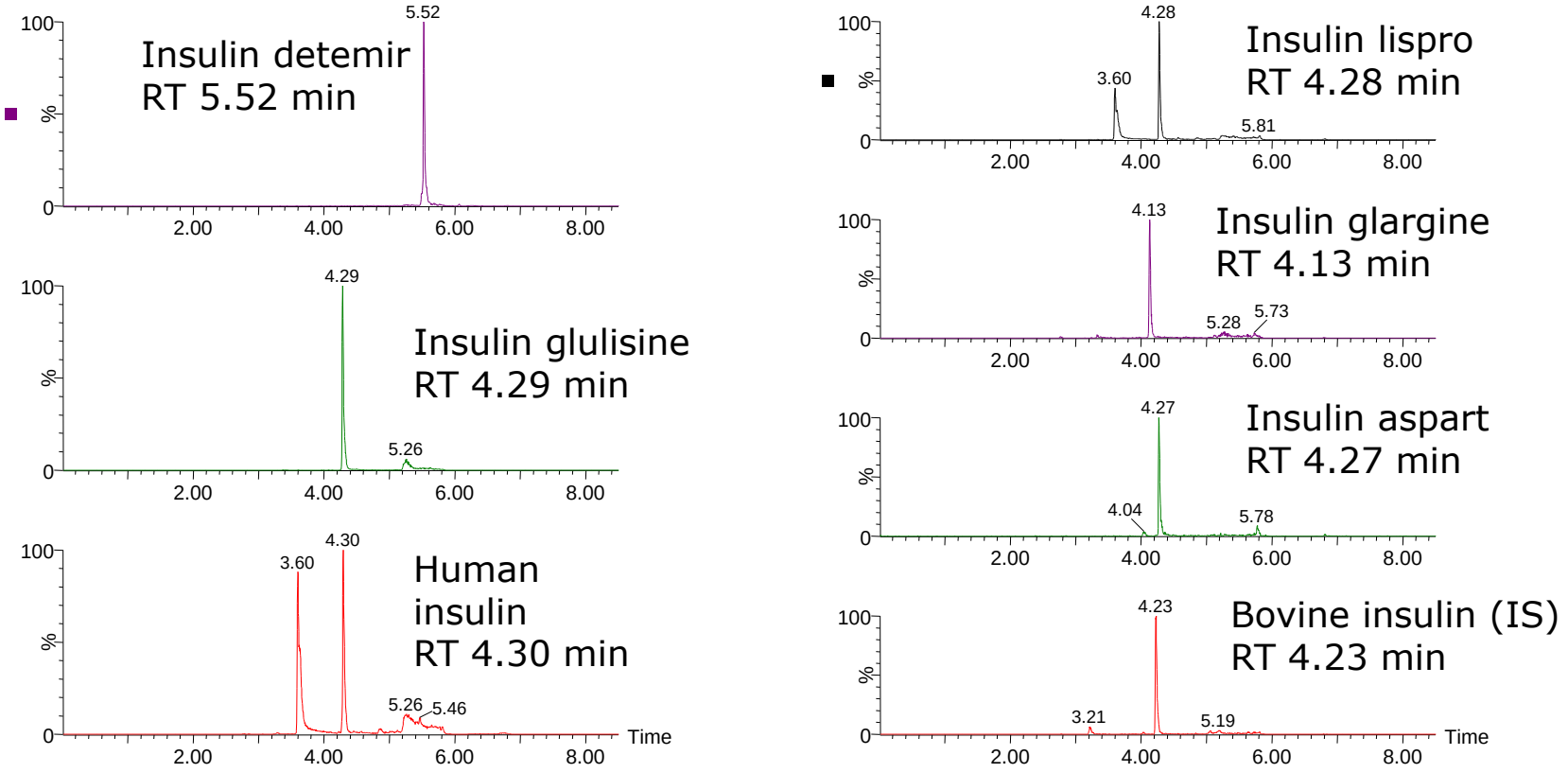

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Extraction Conditions

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PPT followed by Oasis® MAX µElution 96-well plate

- PPT: 250 µL human plasma sample precipitated 1:1 with 50/50 ACN/MeOH + 1% FA, vortex spin 10 min at 13K rcf,
  dilute supernatant with 900 µL 5% NH_4OH in water
- SPE: Oasis® MAX µElution 96-well plate
- Condition: 200 µL methanol
- Equilibrate: 200 µL water
- Load Sample: entire diluted supernatant in 2 steps of ~ 700 µL each
- Wash: 200 µL 5% NH_4OH in water
- Wash: 200 µL 5% methanol, 1% acetic acid in water
- Elute: 2X 25 µL 60% methanol, 10% acetic acid in water
- Dilute: 50 µL water
- Inject 30 µL

Plasma detection limit: 50 pg/mL

Current LOD and LLOQ for Insulin Glulisine in Human Plasma

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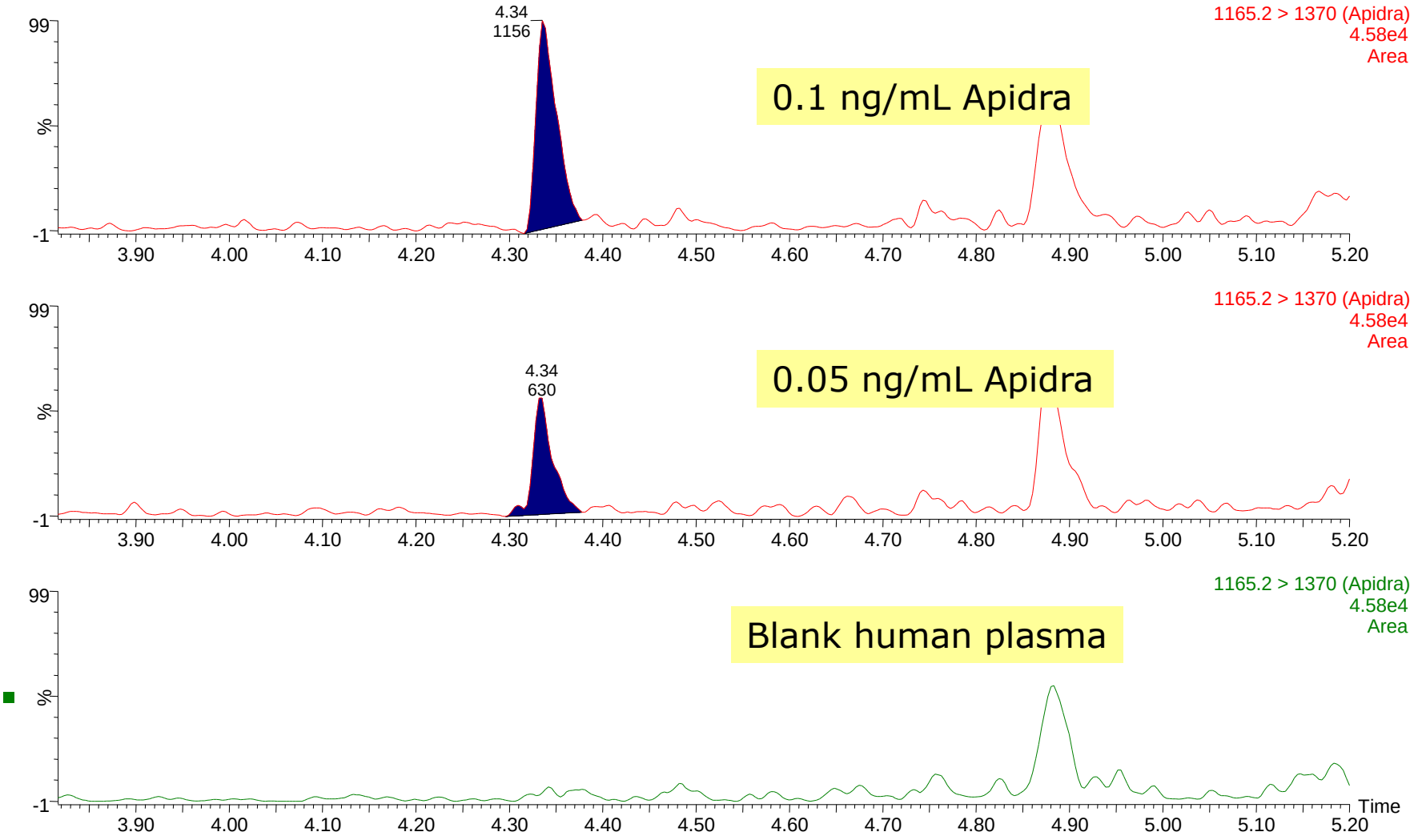

Current Method: Insulin glargine (Lantus) at the LOD and the low QC in Human Plasma

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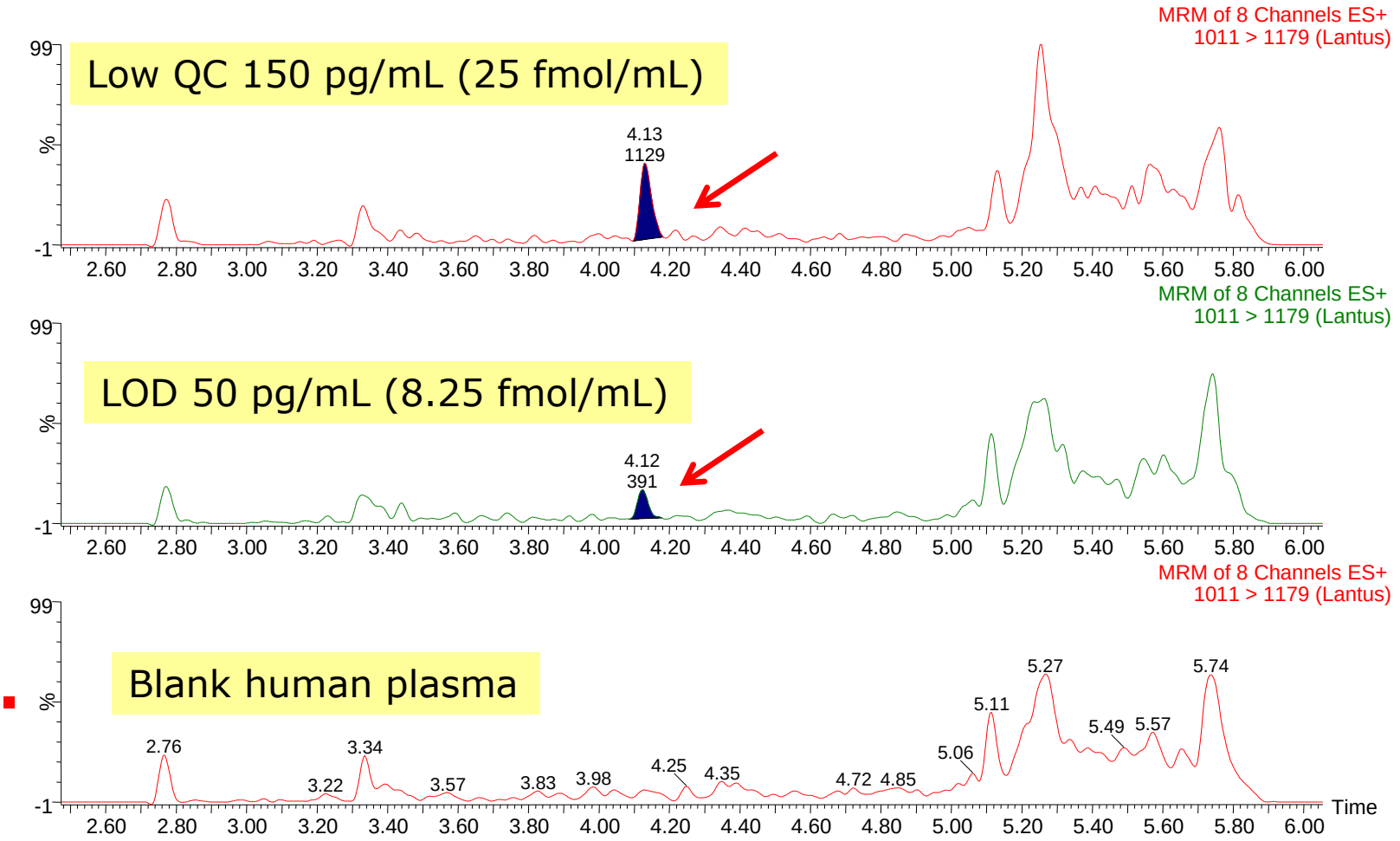
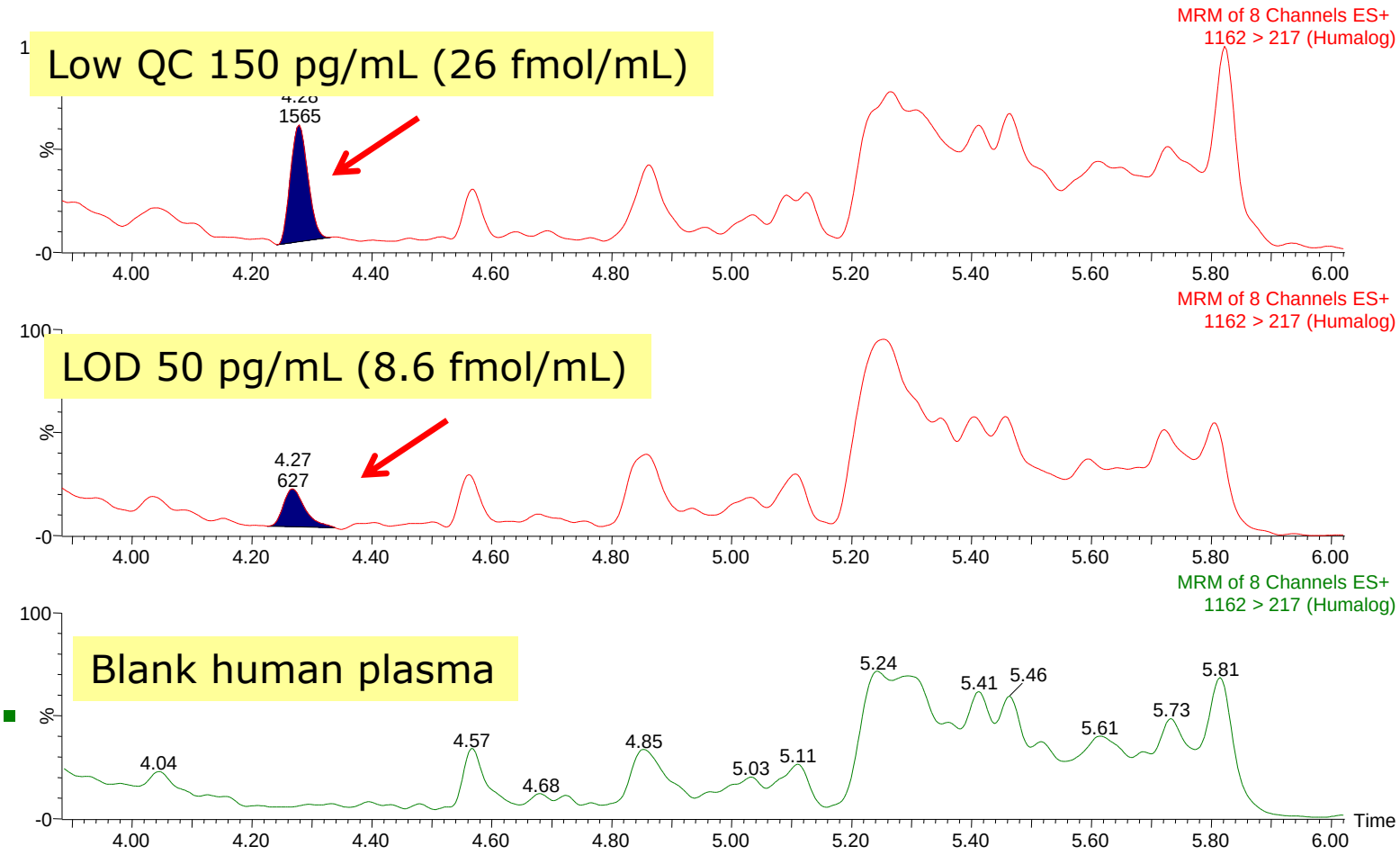


~248 amol on column at LLOQ

Insulin lispro (Humalog) at the LOD and the low QC



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Standard Curve Statistics

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Analyte	Std. Curve Range pg/mL	Std. Curve Range fmol/mL	r^2, linear fit, 1/x weighting	Mean % accuracy of all points
Insulin lispro	50-10,000	8.6-1720	0.998	99.99
Insulin glargine	50-10,000	8.3-1650	0.996	99.98
Human insulin	50-10,000	8.6-1720	0.996	100
Insulin detemir	200-10,000	33.8-1690	0.998	96.4
Insulin glulisine	50-10,000	8.6-1720	0.995	100
Insulin Aspart	100-10,000	17.2-1716	0.995	100

For reference: 1µU/mL = 35 pg/mL = 6 fmol/mL
Volund et al 1991

Human insulin QC Statistics

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Human Insulin — Avg basal level was 1937 pg/mL

Inter-day n=9

QC conc. (pg/mL)	Mean Calc. Conc.	Std Dev	% CV	Mean Accuracy
150	1915.1	125.4	6.5	92.0
750	2542.5	141.0	5.5	94.8
2500	4326.0	146.7	3.4	97.6
7500	9819.0	960.3	9.8	104.0

Intra-day n=3 — Basal level was 1872 pg/mL

QC conc. (pg/mL)	Mean Calc. Conc.	Std Dev	% CV	Mean Accuracy
150	2056.5	16.7	0.8	90.2
750	2506.3	46.6	1.9	99.3
2500	4269.8	206.4	4.8	101.3
7500	10233.2	265.2	2.6	100.3

Insulin lispro QC Statistics

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Insulin Lispro

Inter-day n=9

QC conc. (pg/mL)	Mean Calc. Conc.	Std Dev	% CV	Mean Accuracy
150	144.0	17.5	12.2	96.0
750	721.8	32.3	4.5	96.2
2500	2447.1	202.9	8.3	97.9
7500	7697.5	634.8	8.2	102.6

Intra-day n=3

QC conc. (pg/mL)	Mean Calc. Conc.	Std Dev	% CV	Mean Accuracy
150	164.6	14.9	9.1	109.8
750	748.2	19.8	2.6	99.8
2500	2417.6	230.4	9.5	96.7
7500	8215.4	243.1	3.0	109.5

Insulin glargine QC Statistics

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Insulin Glargine

Inter-day n=9

QC conc. (pg/mL)	Mean Calc. Conc.	Std Dev	% CV	Mean Accuracy
150	150.1	18.7	12.4	102.7
750	718.4	47.3	6.6	95.8
2500	2369.3	131.2	5.5	94.8
7500	7648.5	511.3	6.7	102.0

Intra-day n=3

QC conc. (pg/mL)	Mean Calc. Conc.	Std Dev	% CV	Mean Accuracy
150	167.4	16.6	9.9	111.6
750	757.7	62.4	8.2	101.1
2500	2378.0	184.9	7.8	95.1
7500	7949.5	257.9	3.2	106.0

Further Method Assessment and Implementation

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- Pilot Study with Patient Samples*
  - 22 type I and type II diabetic volunteers
    - Received one or several insulins
  - Dosage regime blind to analytical site
  - Results concur with multi-dosing therapies
- Human insulin over-spike
  - Samples spiked with human insulin at 200X the ULOQ
    - Represent possible high levels expected in diabetics
  - No interference with quantification of any analogs including lispro

*manuscript submitted

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Conclusions/Key Points

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- Detection limits approx. 4X lower (than previous method) for most analogs
  - Only other LC/MS method that reaches these detection limits uses nano-flow and 3-step sample prep involving affinity purification followed by 2 SPE extractions
- The use of the CORTECS C_{18}+ column provided significantly improved sensitivity and peak shape for insulin analogs versus charged-surface fully porous columns and traditional C18 columns
  - Excellent batch-to-batch reproducibility
- 2D LC enables higher loading and further cleanup
- Selective PPT/mixed-mode SPE cleanup significantly reduces endogenous interferences
- Test performed to verify absence of interference when human insulin present at >200X higher concentrations than other analogs
  - For example type II diabetics
  - No cross-talk or impact on quantification observed
- All FDA criteria for accuracy and precision met
  - Average accuracies for standard curve points and QC samples were >92%, with most being close to 99%
  - Inter- and intra-day precision for all QC samples better than 7.5%
  - CV's of matrix factors, for all analogs, across 6 lots of human plasma were <15%

Acknowledgements

Waters Corporation

- Kenneth Fountain
- Stephan Koza
- Thomas Wheat

Kings College London, UK

- Norman Smith
- Cristina Legido-Quigley
- Janaka Karalliedde
- Prof. David Cowan