Hybrid extraction versus physicochemical methods for large peptides: some comparative data and observations

16th EBF Open Symposium

16th November 2023

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2009 – 2020 Insulin Hybrid LCMS Assay
## Immuno-affinity columns and MSIA tips

- **Automated immuno-extraction procedure using capture antibody**

- **Better throughput and more consistent recovery than original manual 'column' method**

- **A hybrid assay combining ligand binding and MS**

- **Applied to clinical studies for several clients**
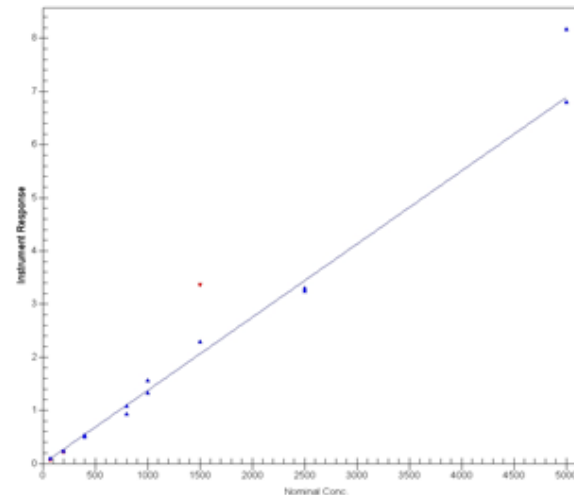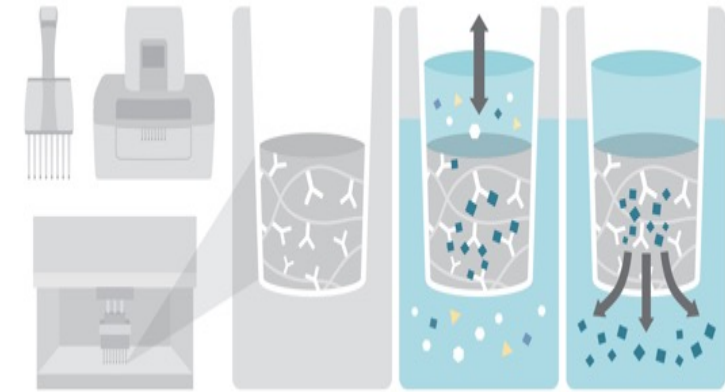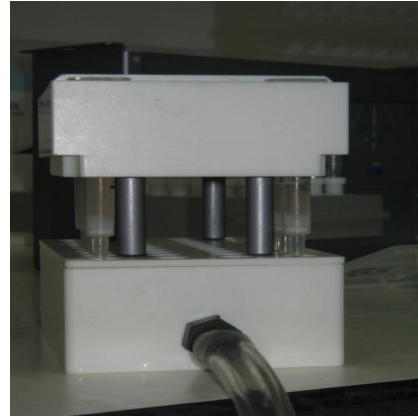
Late 2020 – to date: Physicochemical method

- **From late 2020: MSIA columns no longer in production**

- **No alternative, needed to replace with a more robust process:**
  - *Options: Magnetic beads/Ab, or SPE*
  - *Previous experience with insulins and SPE*

- **2020 – 21 we developed a new method based on physicochemical techniques and 2D LC-MS/MS**
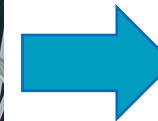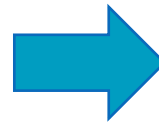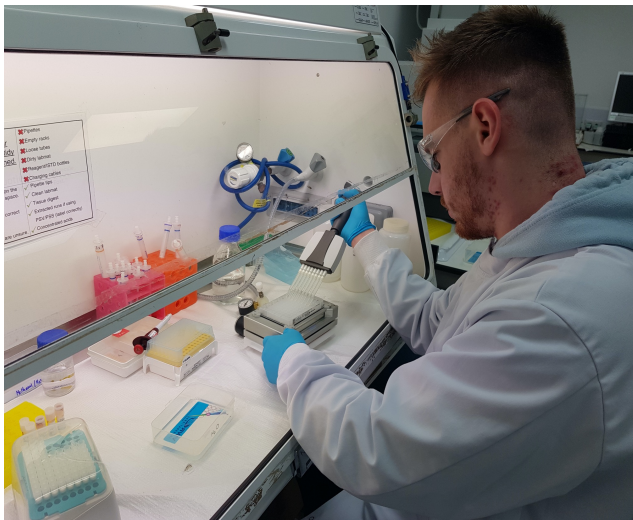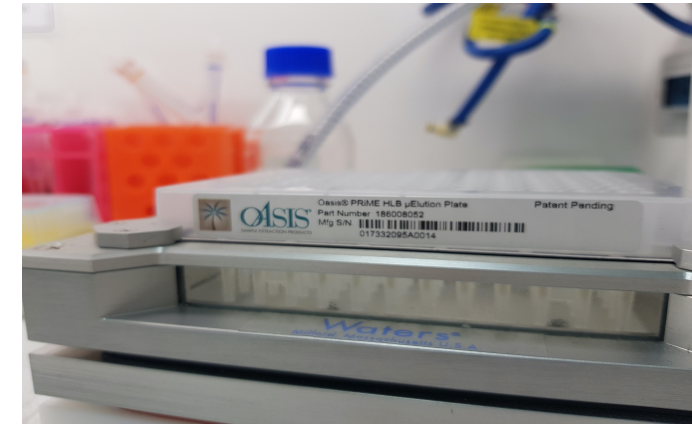
Aims : Hybrid vs SPE Comparison

Develop a method for human insulin using SPE (physicochemical) techniques

Analyse volunteer samples using this new method and compare the data with that from MSIA (hybrid LC-MS)

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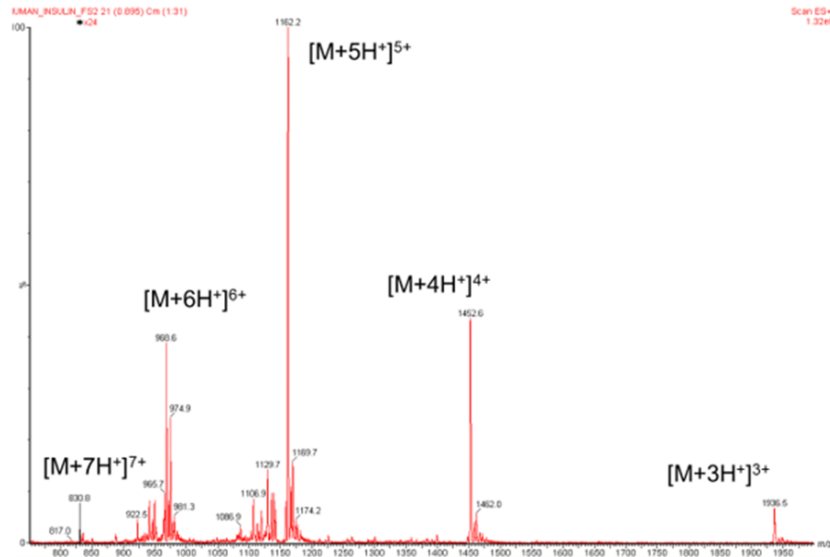
Do the two techniques give the same result for the same sample? Is there a systematic error or bias?
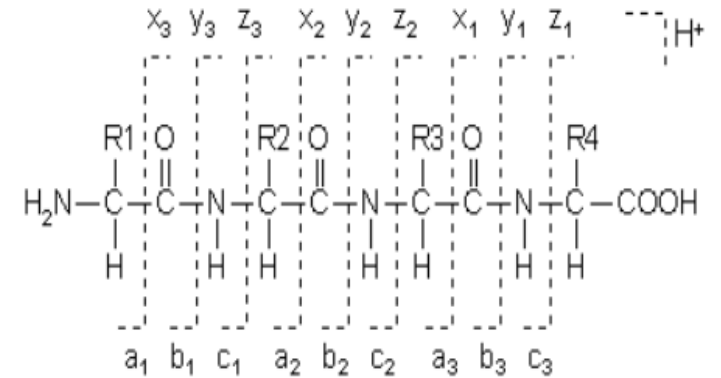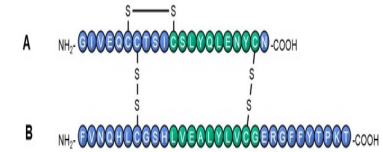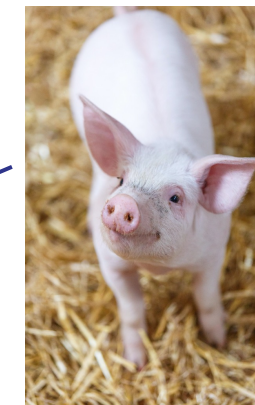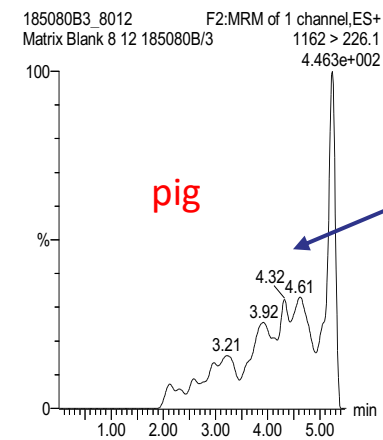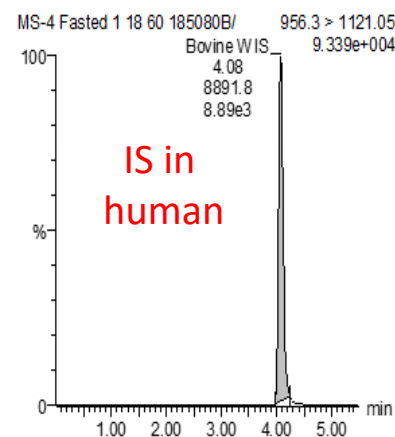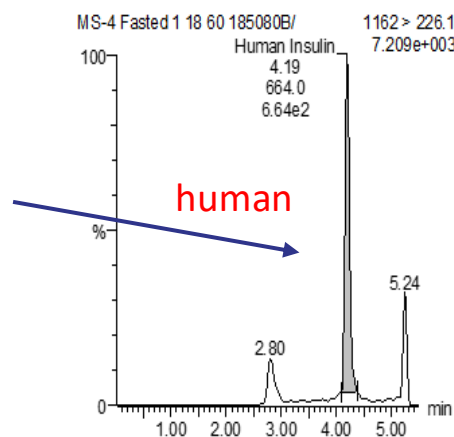
Q1MS   MS - MS
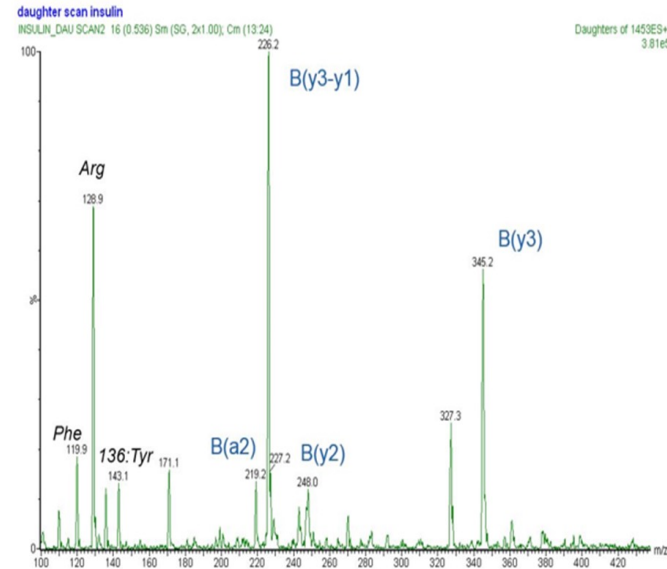
'top down' of intact clusters. Select specific product ion

Human Insulin assay Design

For analogue assays in human, we can simply spike control human plasma with the analogue

Endogenous Human insulin: cannot use same matrix for calibration.

Match with a surrogate matrix: we have chosen pig plasma

Method sample volume is 300 µL

Fed versus Fasted: original test

"The protein concentration is not a meaningful result, unless it is defined which molecular property the method responds to."
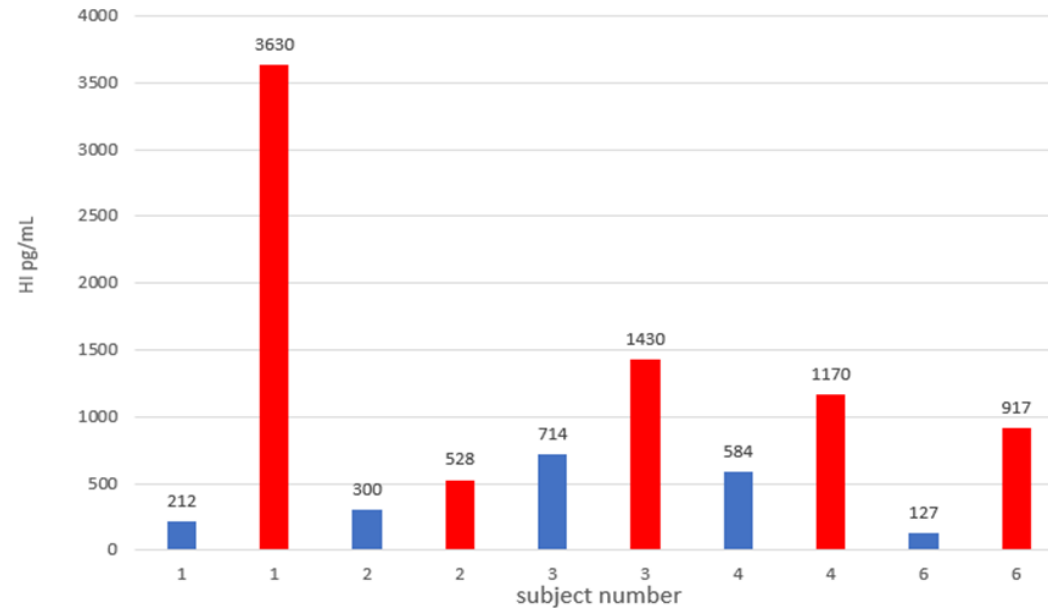*Van der Merbel, European Bioanalytical Forum 2017*

FREE vs. ACTIVE (target binding) vs. TOTAL

MSIA^{TM} extracts target binding insulins, with a capturing epitope on the mid-point of B chain.
To measure TOTAL, crash plasma first & dilute

To prevent non specific binding in the well plate, use a carrier peptide e.g ACTH fragment, leucine enkephalin

Mean fasted and fed (n=6) concentrations of endogenous insulin in 5 healthy volunteers. Values in blue are fasted, red are following a meal.

Calibration Lines Comparison
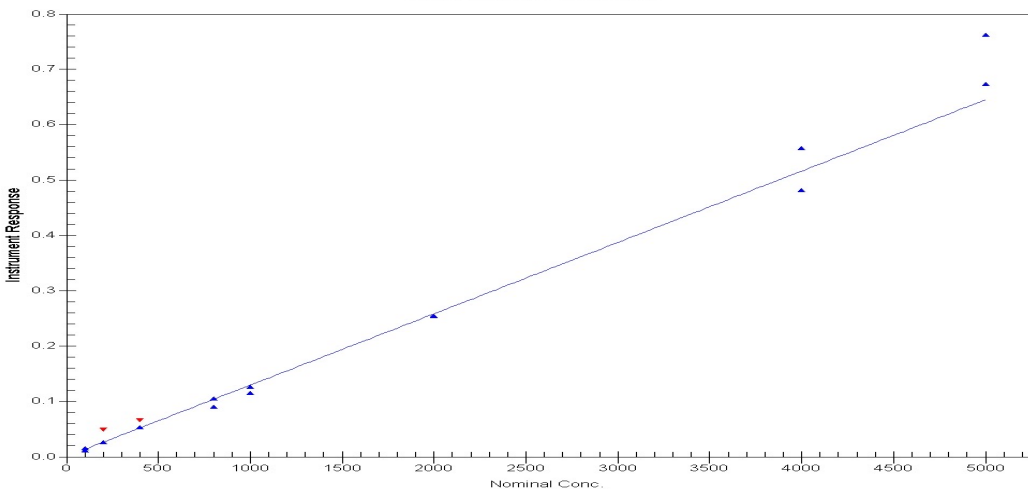
Human insulin spiked pig plasma samples

MSIA extraction

Protein crash followed by SPE (HLB Prime)

2D LC-MS/MS

2D LC-MS/MS

Analytical Run 1 analyzed on 28-Sep-2023 Calibration Standards for Human Insulin (pg/mL)
Regression Method = LINEAR - Weighting Factor = 1/X**2
Response = Slope * Conc + Intercept
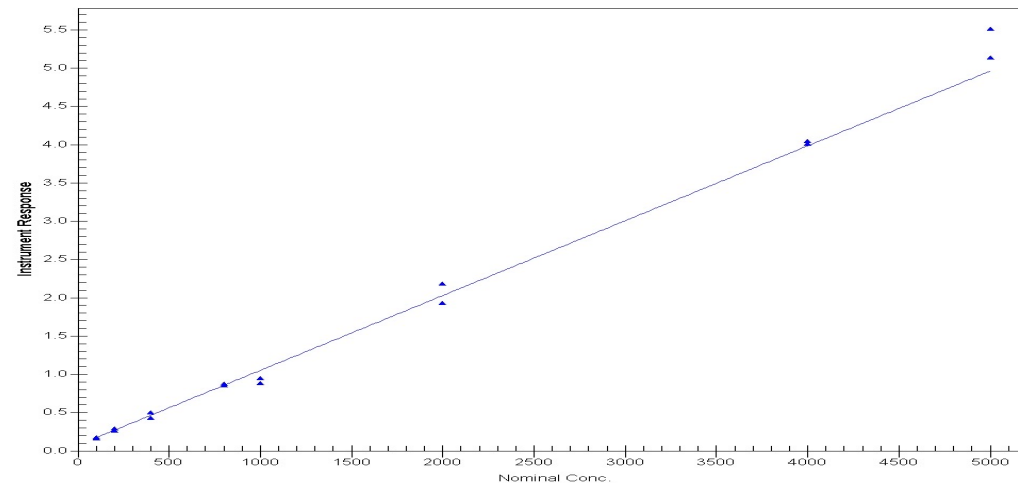Slope = 0.00012882 Intercept = 0.00078647 R-Squared = 0.9862
(Study MSIA_SPE_CROSSVAL)

Analytical Run 2 analyzed on 28-Sep-2023 Calibration Standards for Human Insulin (pg/mL)
Regression Method = LINEAR - Weighting Factor = 1/X**2
Response = Slope * Conc + Intercept
Slope = 0.00097773 Intercept = 0.074272 R-Squared = 0.9894
(Study MSIA_SPE_CROSSVAL)
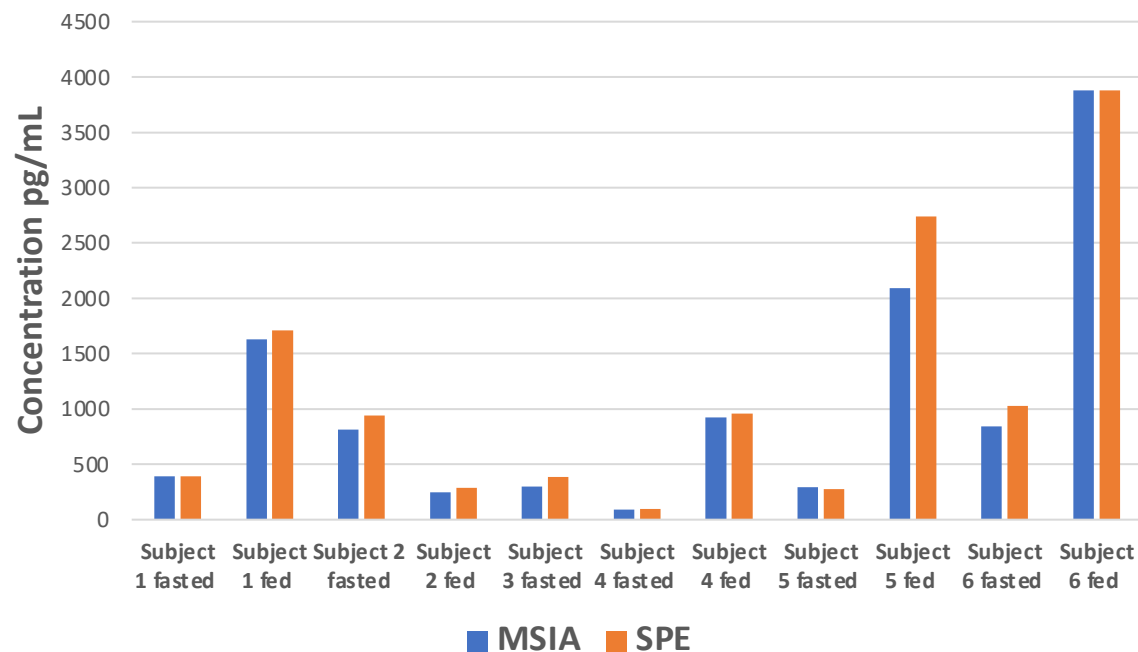
MSIA
2 points excluded

SPE
No points excluded

Fed and fasted volunteers
MSIA versus SPE

All Concentrations pg/mL

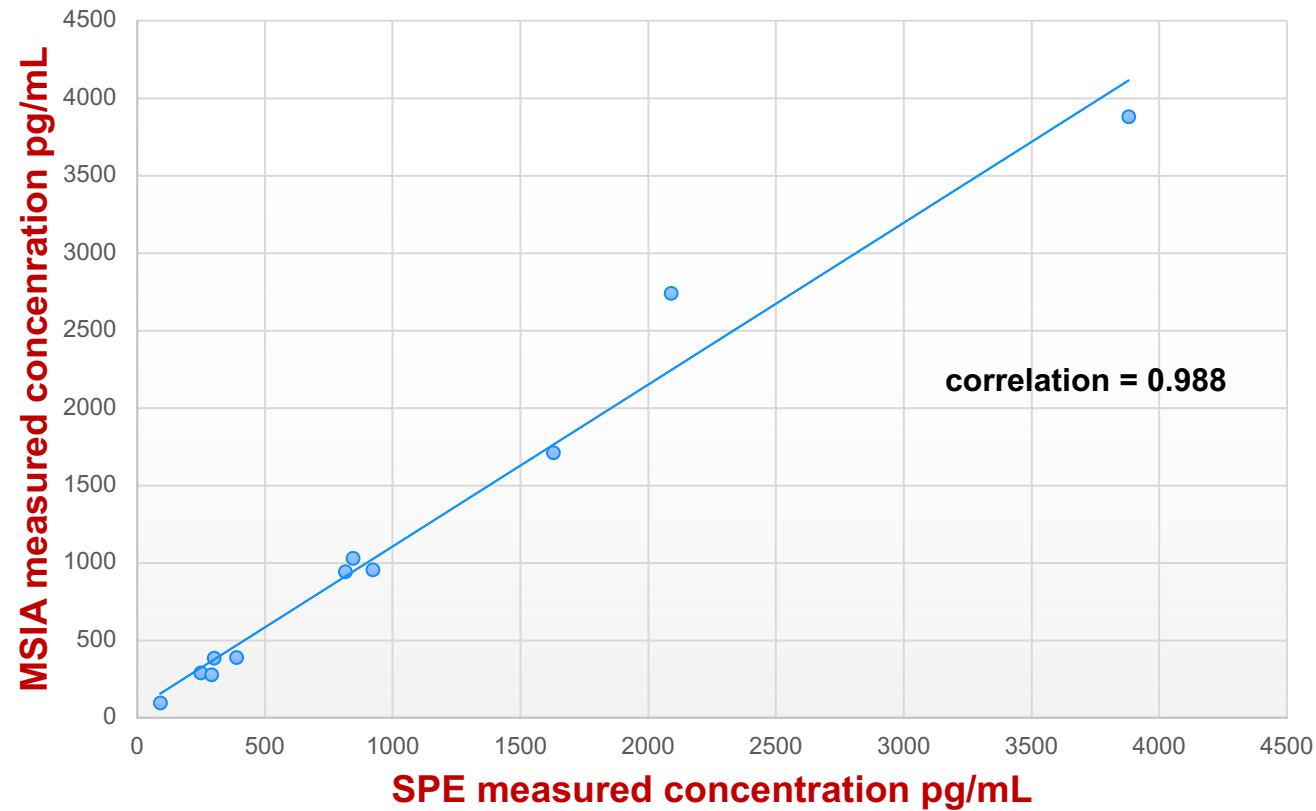| Sample	MSIA	SPE
Subject 1 fasted	390	389
Subject 1 fed	1630	1710
Subject 2 fasted	815	941
Subject 2 fed	248	288
Subject 3 fasted*	300	383
Subject 4 fasted	89.6	97.7
Subject 4 fed	923	957
Subject 5 fasted	292	278
Subject 5 fed	2090	2740
Subject 6 fasted	845	1030
Subject 6 fed	3880	3880

*removed blocked
MSIA Tip (3, fed)

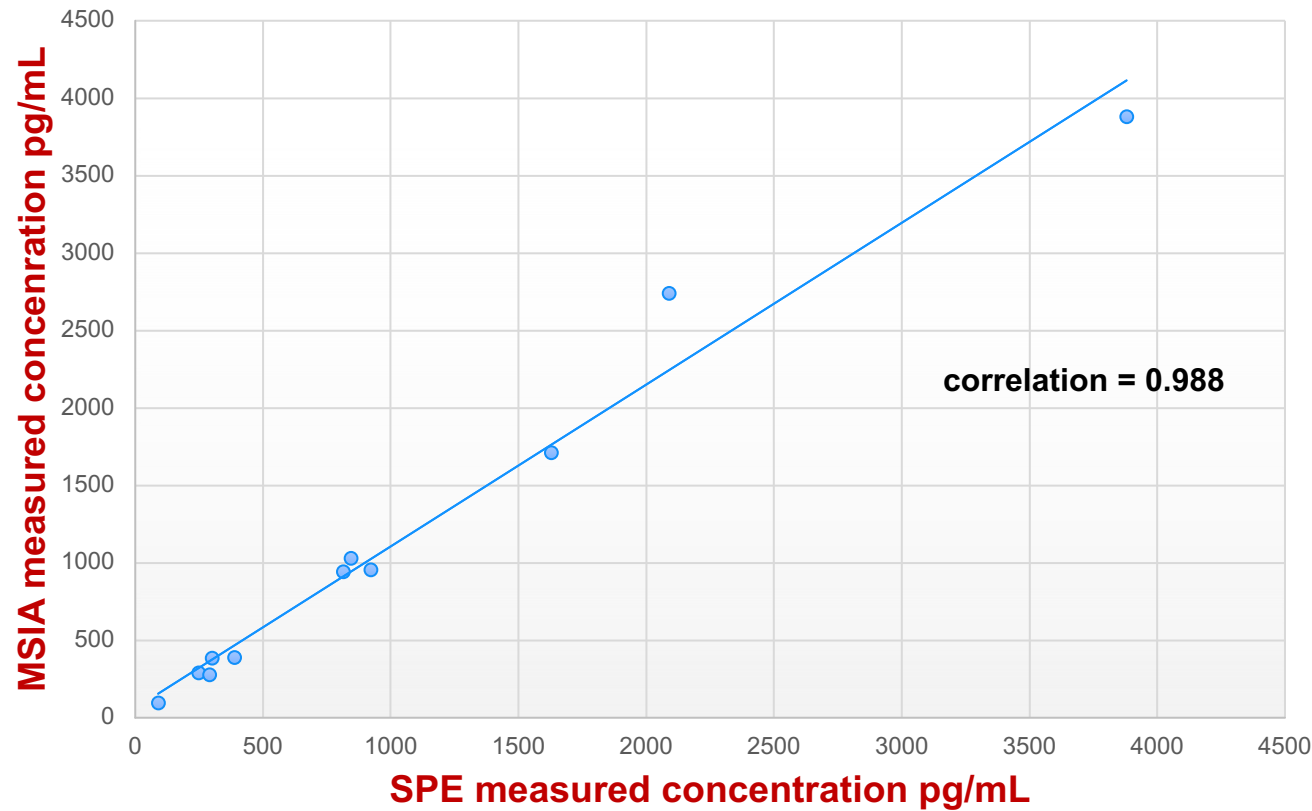### Fed & Fasted Human Insulin MSIA versus SPE

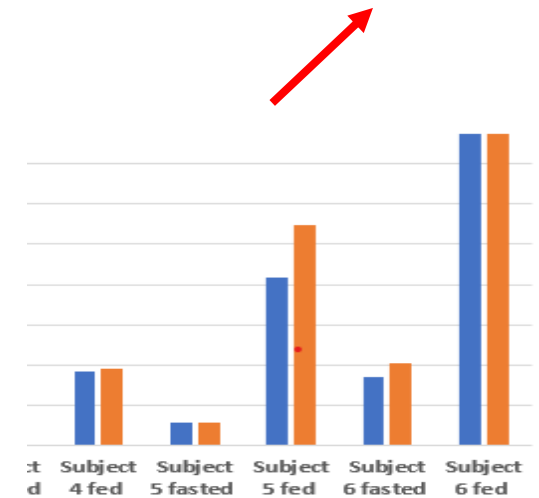# Correlation of SPE versus MSIA

Scatter Chart MSIA vs SPE

correlation = 0.988

SPE measured concentration pg/mL

MSIA measured concenration pg/mL

Correlation of SPE versus MSIA

Scatter Chart MSIA vs SPE

correlation = 0.988

X-axis: SPE measured concentration pg/mL
Y-axis: MSIA measured concenration pg/mL

Average MSIA value	Average SPE value
1046	1154
-	+ 10.3%

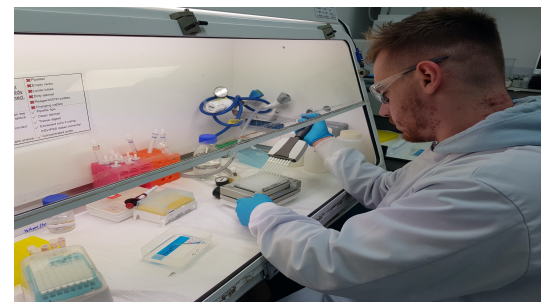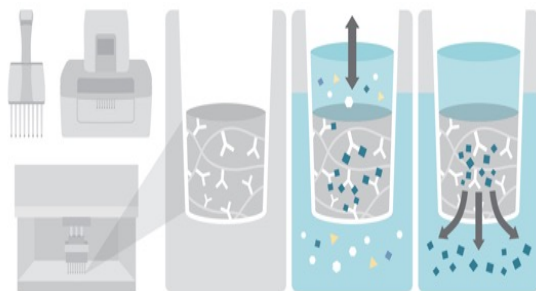Subject 4 fed | Subject 5 fasted | Subject 5 fed | Subject 6 fasted | Subject 6 fed

Acceptance Criteria
Inconsistency

LBA 20/25

1 step

SPE 15/20

3 steps

2 methods generating equivalent data, same PK endpoint but different acceptance.

Reference: Immunocapture LC/MS(/MS) assays for biotherapeutic and biomarker proteins – the European Bioanalysis Forum continuing discussions on scientific and regulatory challenges. Barfield et al., Bioanalysis Vol. 15 No 9. White Paper

High Mass Product Ions
for greater specificity
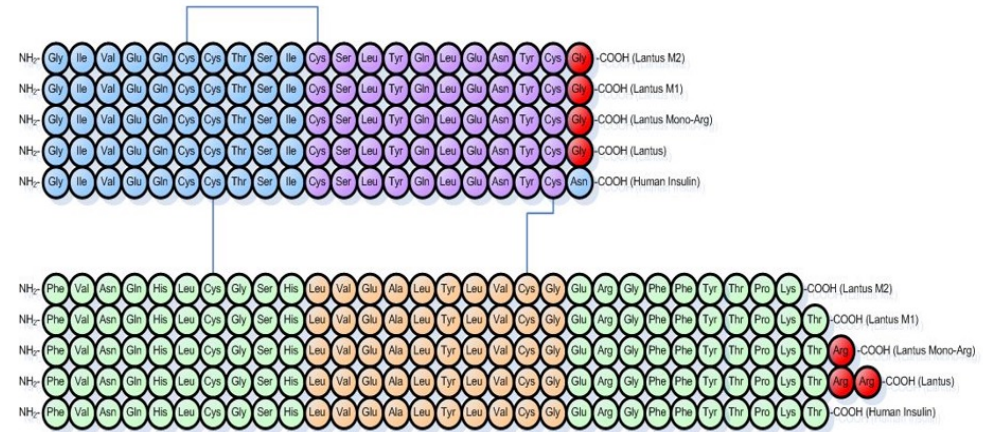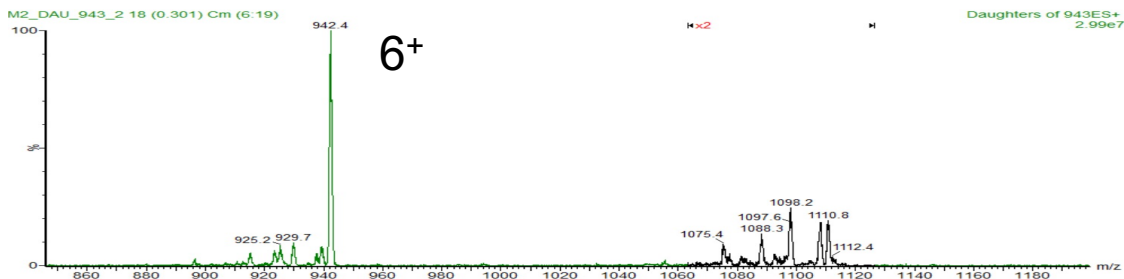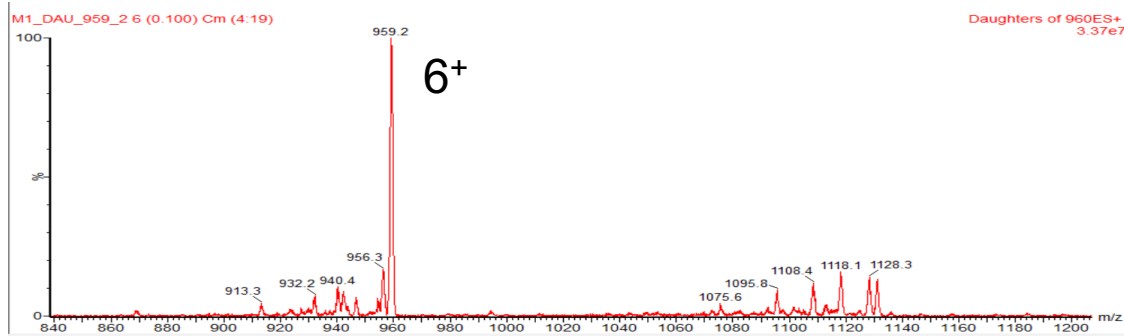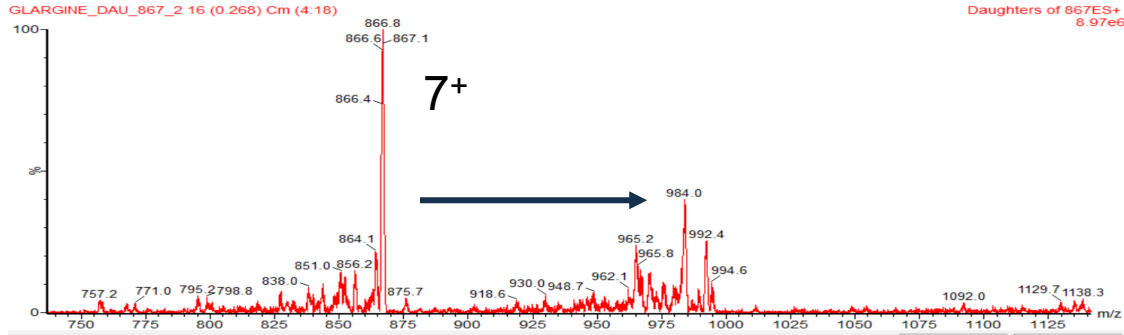
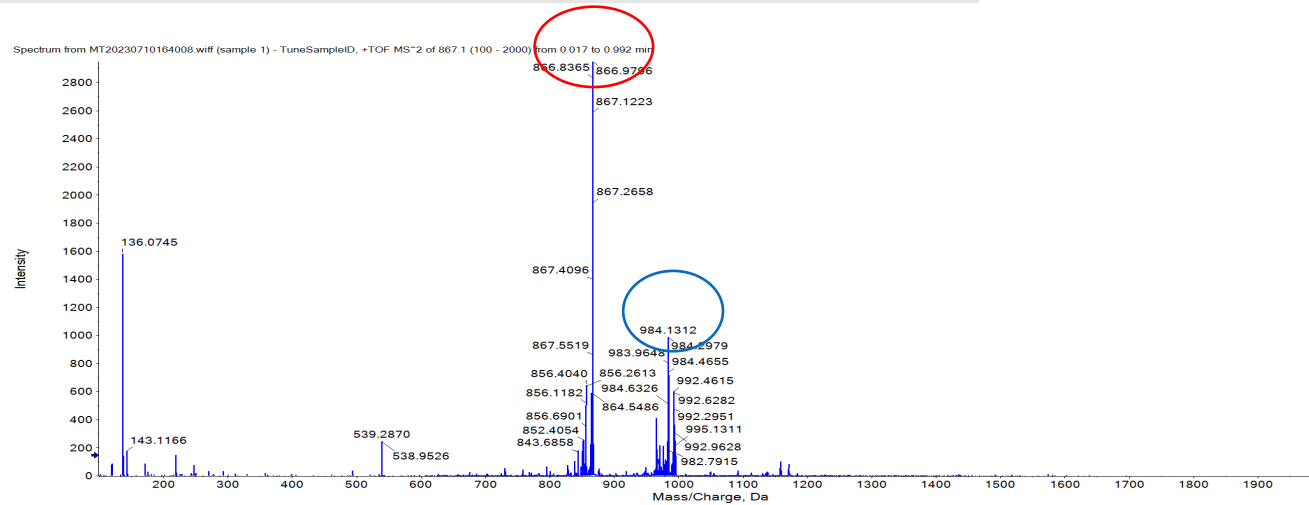| Compound	Glargine	M1	M2	Lispro	Bovine IS
Hybrid	867>136 Tyr	959>226 By3-y1	943>136 Tyr	969>217 By2	956>1121 *
SPE	867>984 A+y19B?	959>1108 *	943>1098 *	969>217 By2	956>1121 *
	1011>1179				

Small product = high collision energy 35-50eV
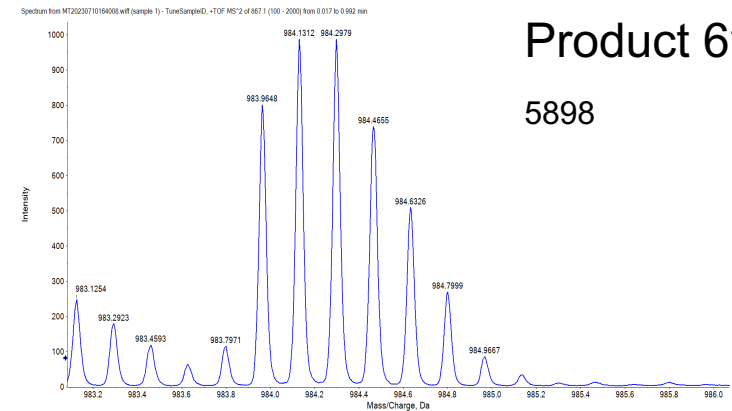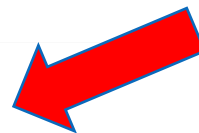Large product = lower collision energy

Glargine
Identity of high mass product ions: HR-MS

Spectrum from MT20230710164008.wiff (sample 1) - TuneSampleID, +TOF MS^2 of 867.1 (100 - 2000) from 0.017 to 0.992 min

6061 glargine
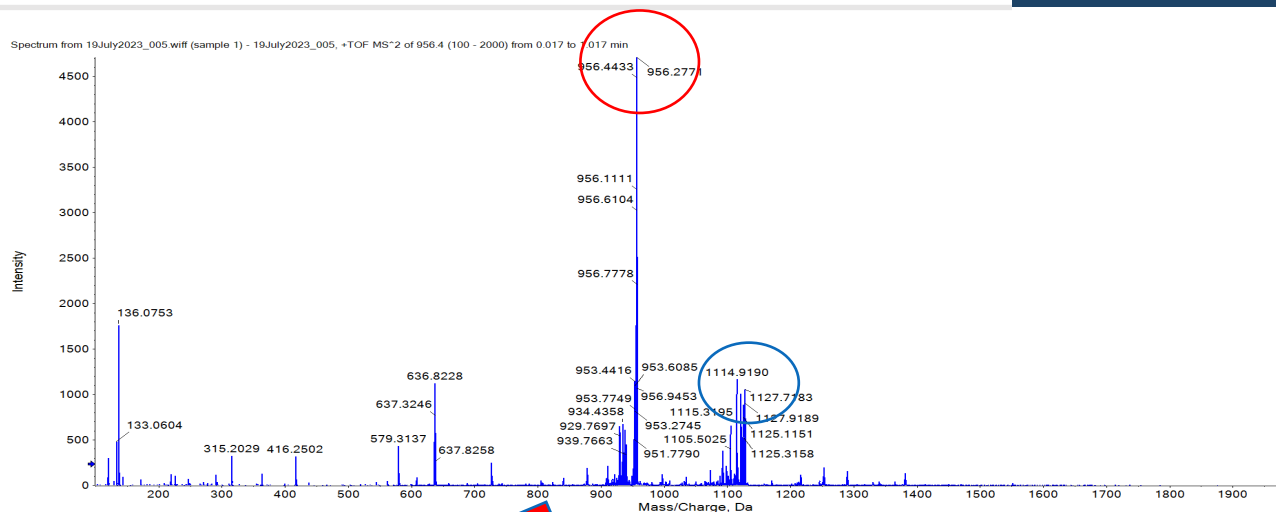## Precursor 7$^+$
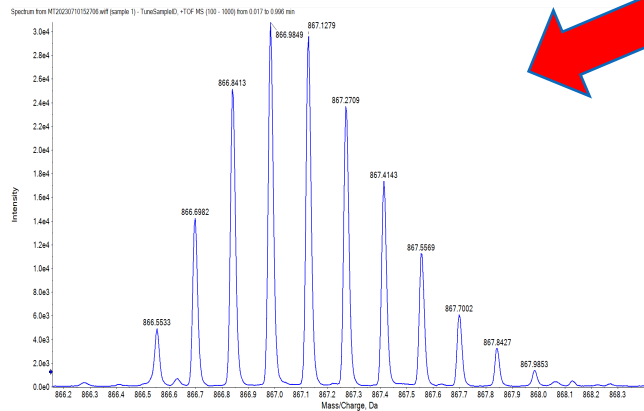
Product 6$^+$

5898

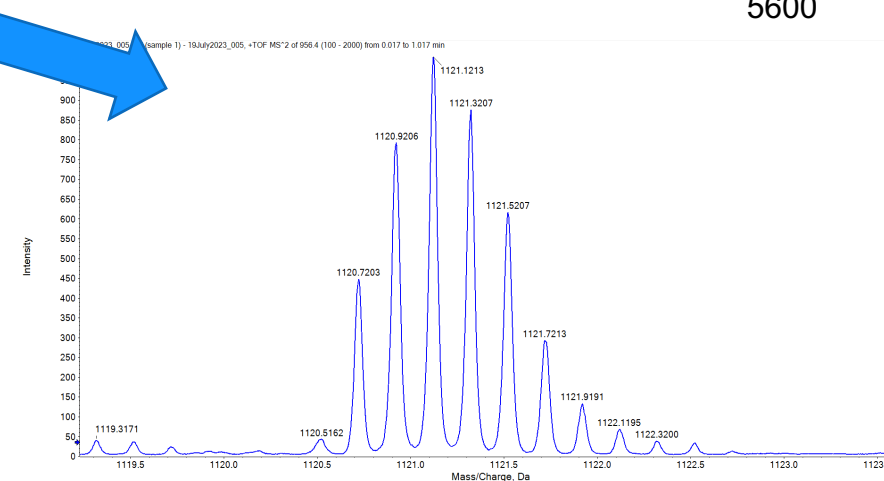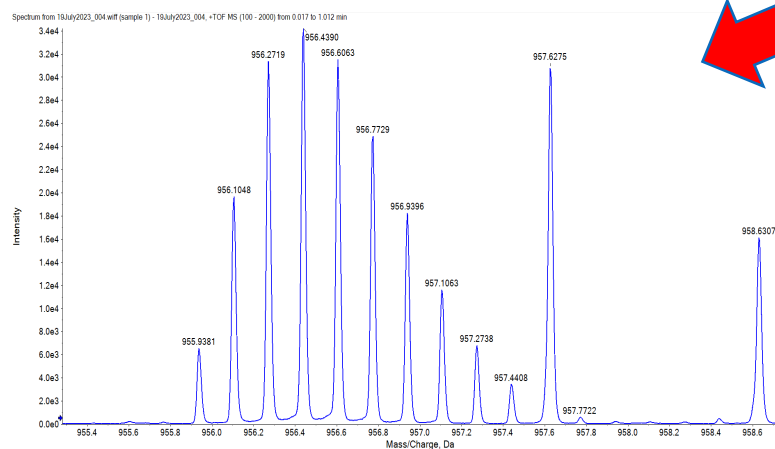**Accurate mass consistent with the loss of 9 water molecules**

Bovine IS
Identity of high mass product ions HR-MS

Precursor 6+
5732

Product 5+
5600

tentatively the precursor ion loses asparagine from the C-terminal position of the B-chain
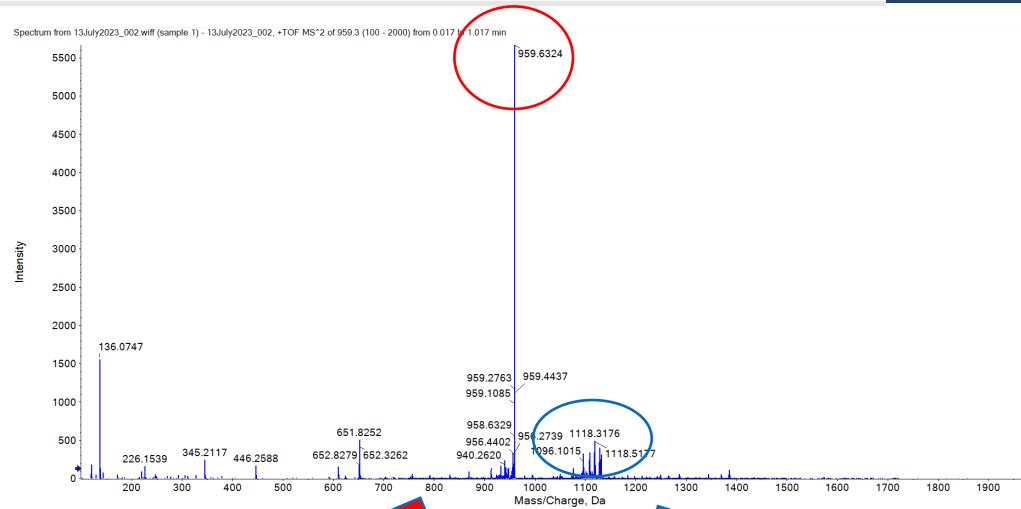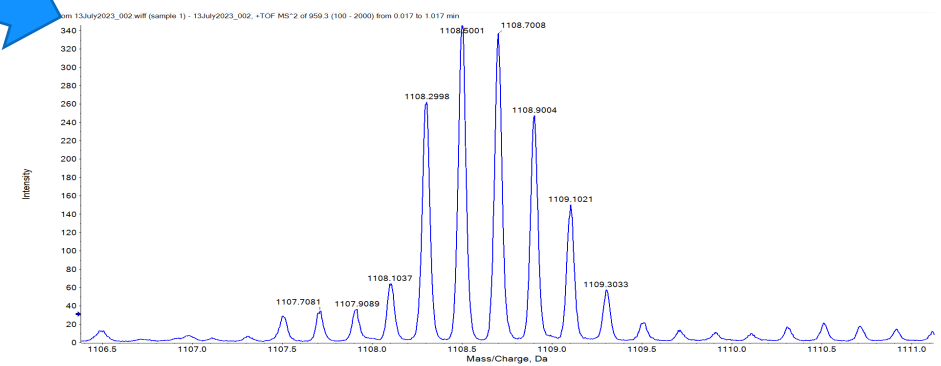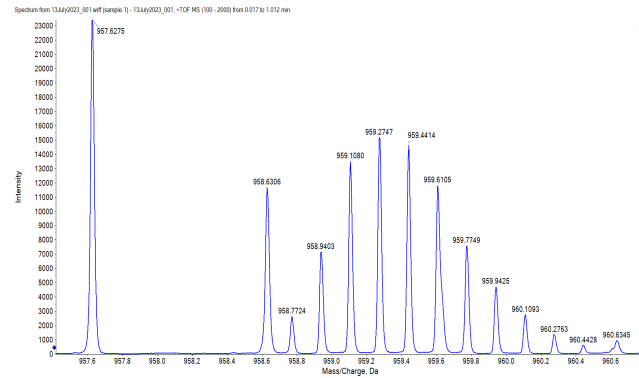
Glargine M1 HR-MS
No product assignment so far

Precursor 6⁺
5750

Product 5⁺
5536

Summary

✓   Transferred a method for human insulin from hybrid to physicochemical format to test equivalence between assay types

✓   Ran fed and fasted plasma samples from six site volunteers through each method to compare the results

✓   The results were equivalent and correlated well. No significant bias between hybrid LC-MS and SPE LC-MS methods. Acceptance criteria?

✓   Many peptide assays use product ions > mass than parent for added specificity. Tentative ids for glargine and bovine insulin

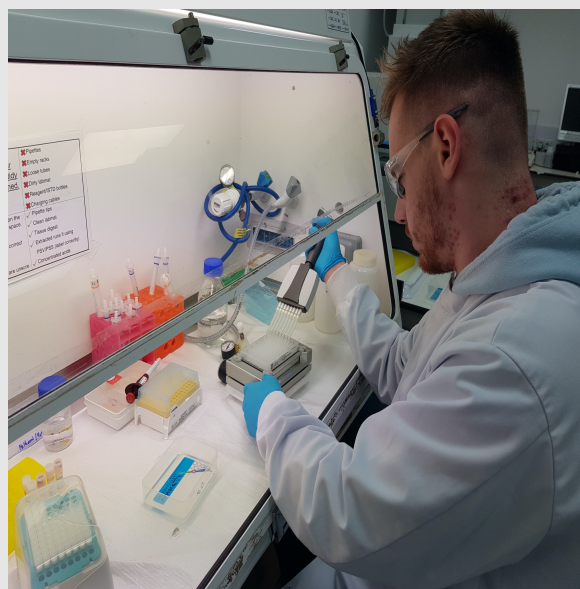✓ Relatively low collision energy, specificity as these are high mass products derived from simple losses

Acknowledgements

Assay: Danny Horton, Janine Morsman, Stephen Gray

Matrix: Miranda Wilkinson, Fiona Holden, six blood volunteers

THANK YOU FOR LISTENING!