Challenges in peptide mapping mass spectrometry of biopharmaceuticals

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Inspired by patients.
Driven by science.

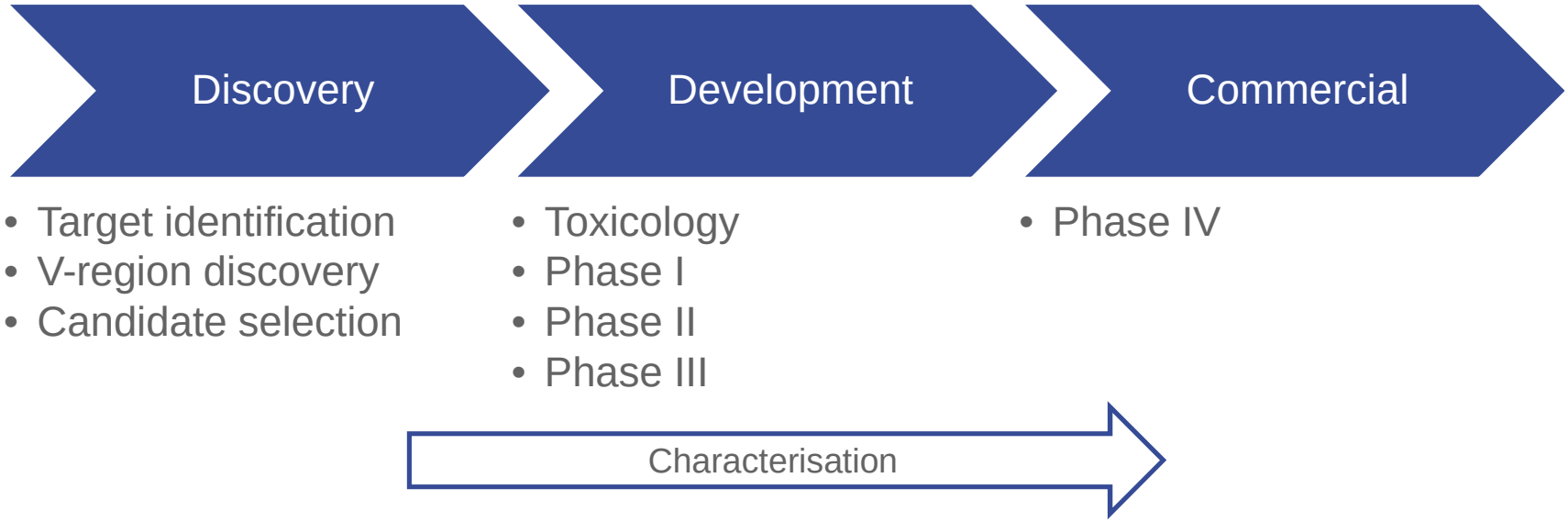
Characterisation within UCB

Context to the type of analysis performed

UCB is a global biopharma focused on severe diseases in two therapeutic areas: neurology and immunology

Characterisation is on the biology side of the business:

- **Biopharmaceuticals rather than small molecules**
- Antibody based molecules produced in cell culture (mammalian and e-coli)



Characterisation within development

Who do we work with and what do we do?

Activities with in development:

Process scale-up and optimisation

- Upstream (cell-culture)
- Down stream purification

Formulation

Product understanding

Analytical

- Method development
- Stability
- Bioassay

Types of study:

- Comparability
- Reference standard characterisation
- Process understanding
- Force degradation study
- Stability
- Investigations

What types of analysis do we perform?

Wide range of physical/biophysical

Gel separations

- SDS-PAGE
- CE

HPLC separations

- Cation exchange
- Size exclusion

Higher Order

- CD, FTIR, DSC, AUC, Biacore, DLS

Mass spectrometry

- Peptide mapping reduced and non-reduced
- Intact mass denatured and native

Gives information on the primary structure

What people want to know…

- Expected sequence confirmation
- PTM levels
- Sequence variants

Peptide mapping

Method

Sample preparation (digestion)

- Denature (guanidine hydrochloride)
- Reduce (dithiothreitol)
- Alkylate (iodoacetamide)
- Buffer exchange
- Digest (trypsin for 3 hours)

Separation

- Reversed phase C_{18}
- Water:acetonitrile with 0.1% formic acid
- 45 minute run time

Detection

- Q-Exactive Plus Orbitrap

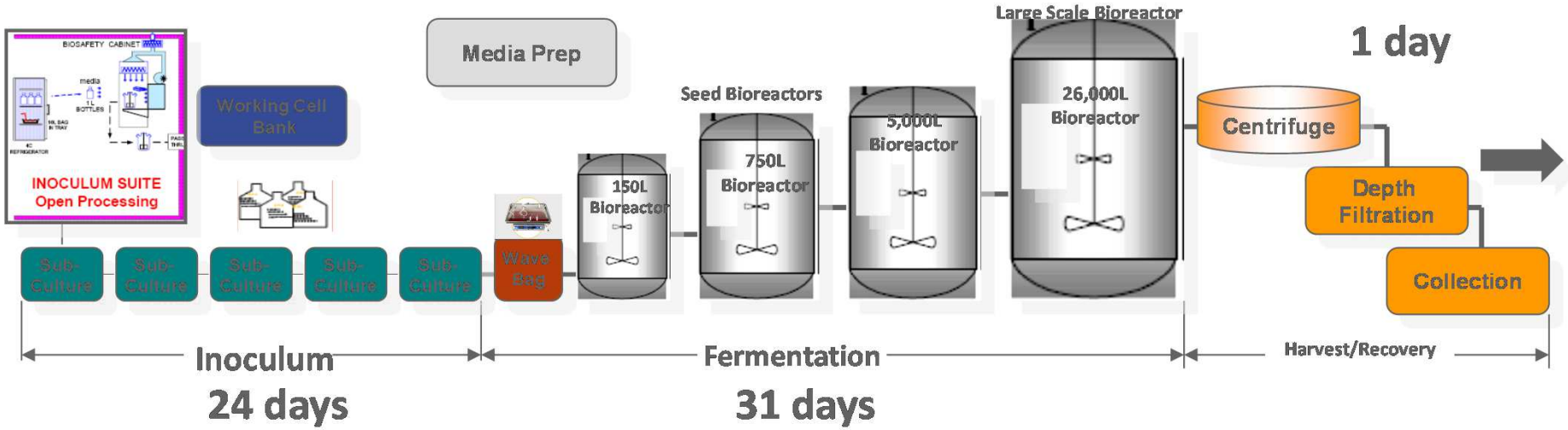
Data processing

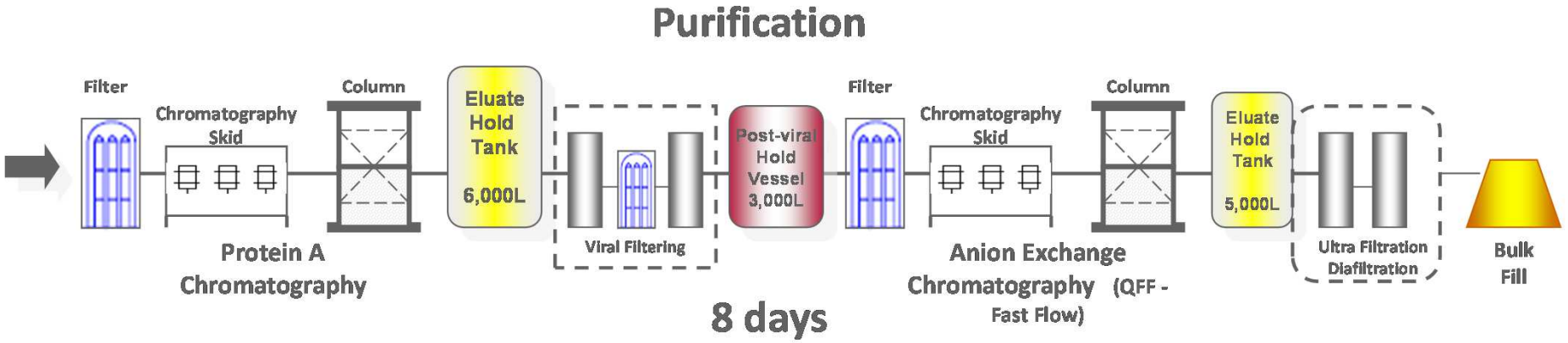
- Biopharama Finder

Ideal method:

- Reproducible
- Not induce PTMs during analysis
- Scalable
- Fast
- Specific cleavage
- Sensitive

Manufacture overview of a biopharmaceutical





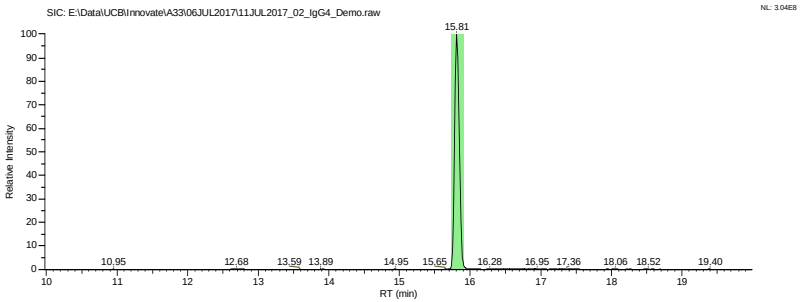
Modification levels

How are these calculated?

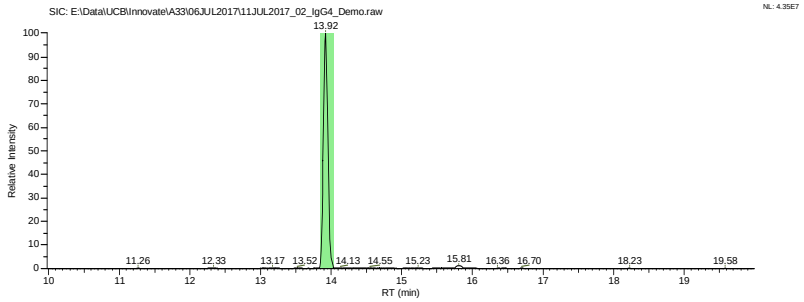
Example of how the level at which a PTM is present is calculated (oxidation)

Peptide: DTLMISR

Non-modified selected ion chromatogram:



Oxidised selected ion chromatogram:



Easy example:

- One modification site
- Good chromatographic separation
- 16 Da mass shift

To calculate oxidation level:

Integrate peak area of non-modified and modified

Not quantitative

Can be used to compare levels across batches within the same sample set

$$\text{Modification Level} = \frac{Peak\ area\ of\ modified}{Peak\ area\ of\ (modified + non-modified)}$$

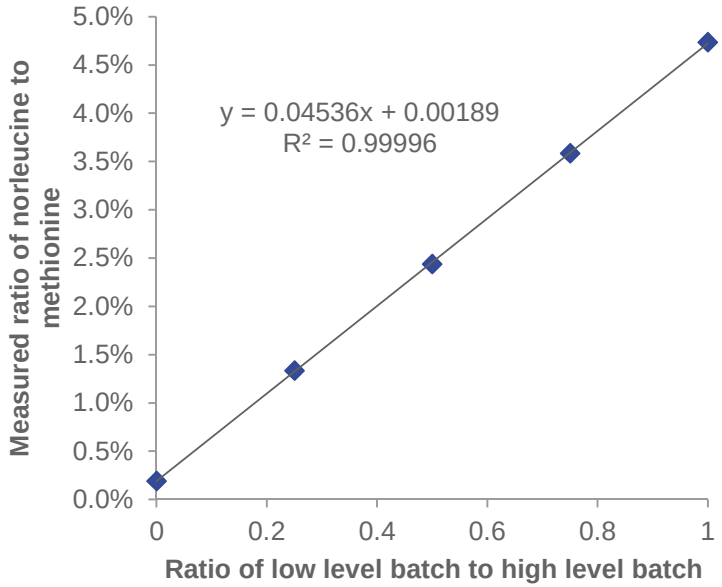
Example of measurement of modification level

Easy example norleucine misincorporation for methioninine

Repeatability

	Lower concentration at about 1.3%		Repeat injections at about 2.3%		Higher concentrations at about 4.4%	
	Std Dev	*CV*	*Std Dev*	*CV*	*Std Dev*	*CV*
Norleucine level	0.06%	3.6%	0.05%	2.0%	0.13%	2.6%

Linearity



Method can give linear and repeatable results

Modifications searched for

How many should be included?

Standard modifications:

- Glycosylation (glycoforms)
- C-terminal lysine clipping
- N-terminal pyroglutamic acid formation
- Methionine oxidation
- Deamidation
- Glycation

Additional modifications:

- Tryptophan oxidation
- Histidine oxidation
- O-linked glycosylation
- Aspartic acid isomerisation
- Glycation degradation products (AGEs)
- Leader sequence
- Sequence variants
- …
- Non-hypothesis modifications (wild card search)

What are the issues with this approach?

Levels reported are not quantitative

- Comparison between runs and instruments is difficult
- Comparison between occasions can be difficult
- How should levels be calculated?

When is a sample different

- Analytical difference Vs biochemically relevant difference

Detection of repeated sequences

- Would the presence of additional light chain be detected?

Too much data

- Large numbers of samples
- How to spot relevant differences quickly
- Which differences relate to the various parameters?

What improvements are we trying to make?

Two separate approaches

Isotopically labelled products

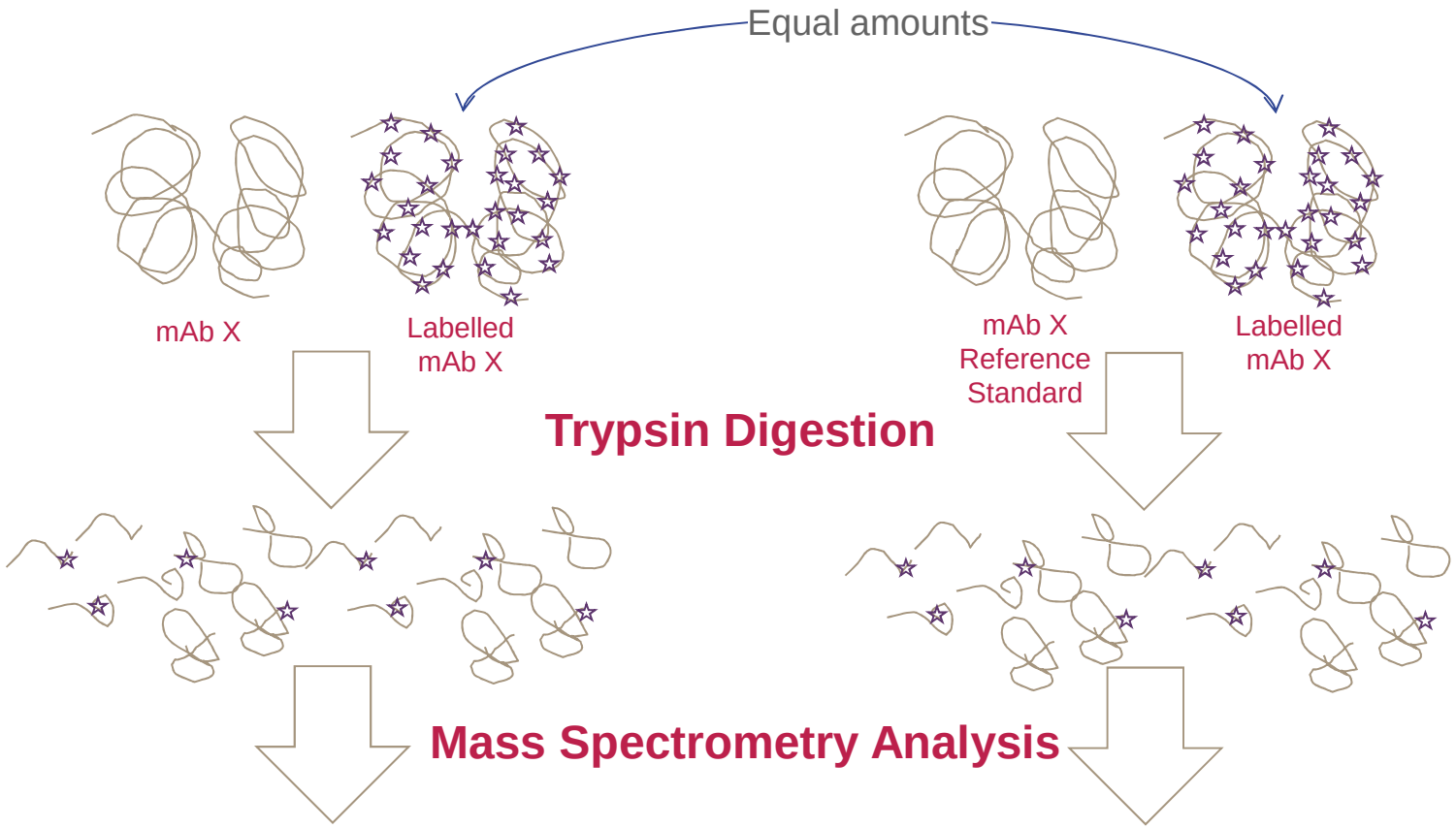
- For each product make a small amount that has been isotopically labelled so that this can be used as an internal standard.
- Focus on the amount of unmodified peptide present
- Any modification of a peptide will reduce the amount of unmodified peptide
- Doesn’t matter if you know what modifications to look for

Statistical methods

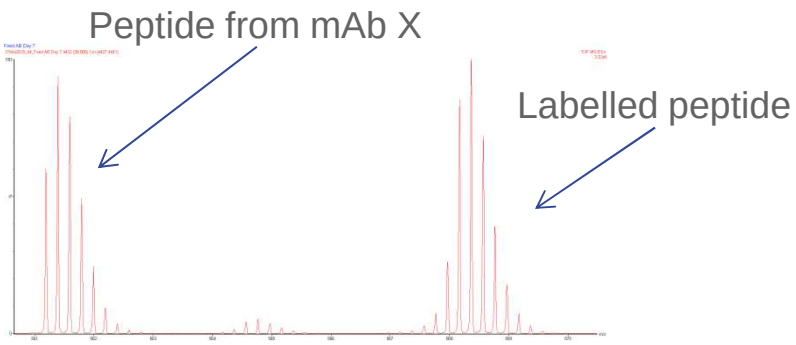
- Focus only on the PTMs and the levels that have been measured for each
- Takes some account of loading and digestion differences
- Include all modifications: hypothesis/non-hypothesis and sequence variants

Labelled internal standard methodology

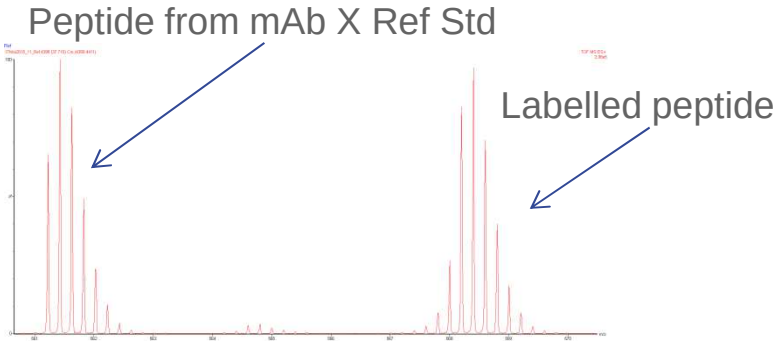
Principle



Labelled internal standard methodology (2)



A B



C D

Measure intensity of peptide signals:

I_A = Intensity of peptide from mAb X
I_B = Intensity of labelled peptide in mAb X sample

I_C = Intensity of peptide from Ref Std
I_D = Intensity of labelled peptide in Ref Std sample

Calculate ratio of peptide and labelled peptide in each sample:

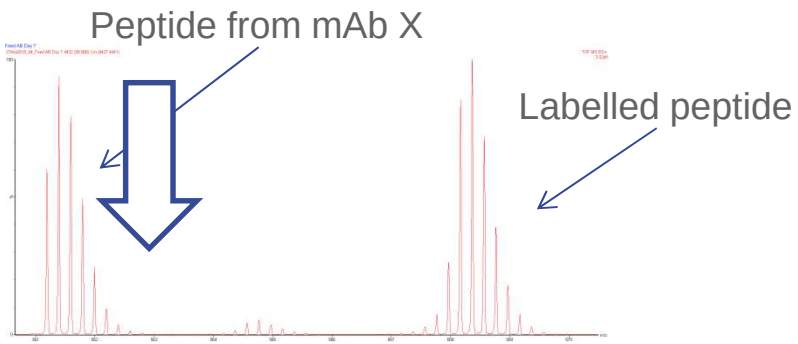
$$\frac{I_A}{I_B} \qquad \frac{I_C}{I_D}$$

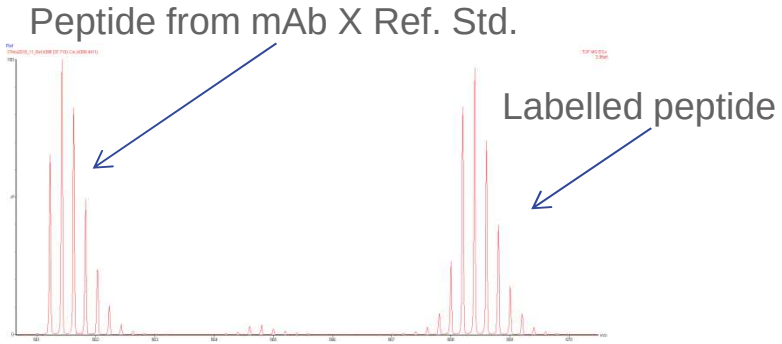
Calculate ratio of ratios to give the amount of the mAb X peptide relative to the mAb X Ref. Std. peptide:

$$\frac{\frac{I_A}{I_B}}{\frac{I_C}{I_D}} = \frac{I_A}{I_C}\frac{I_D}{I_B} = ratio\ of\ sample\ to\ standard$$

Labelled internal standard methodology (3)

Differences





A B C D

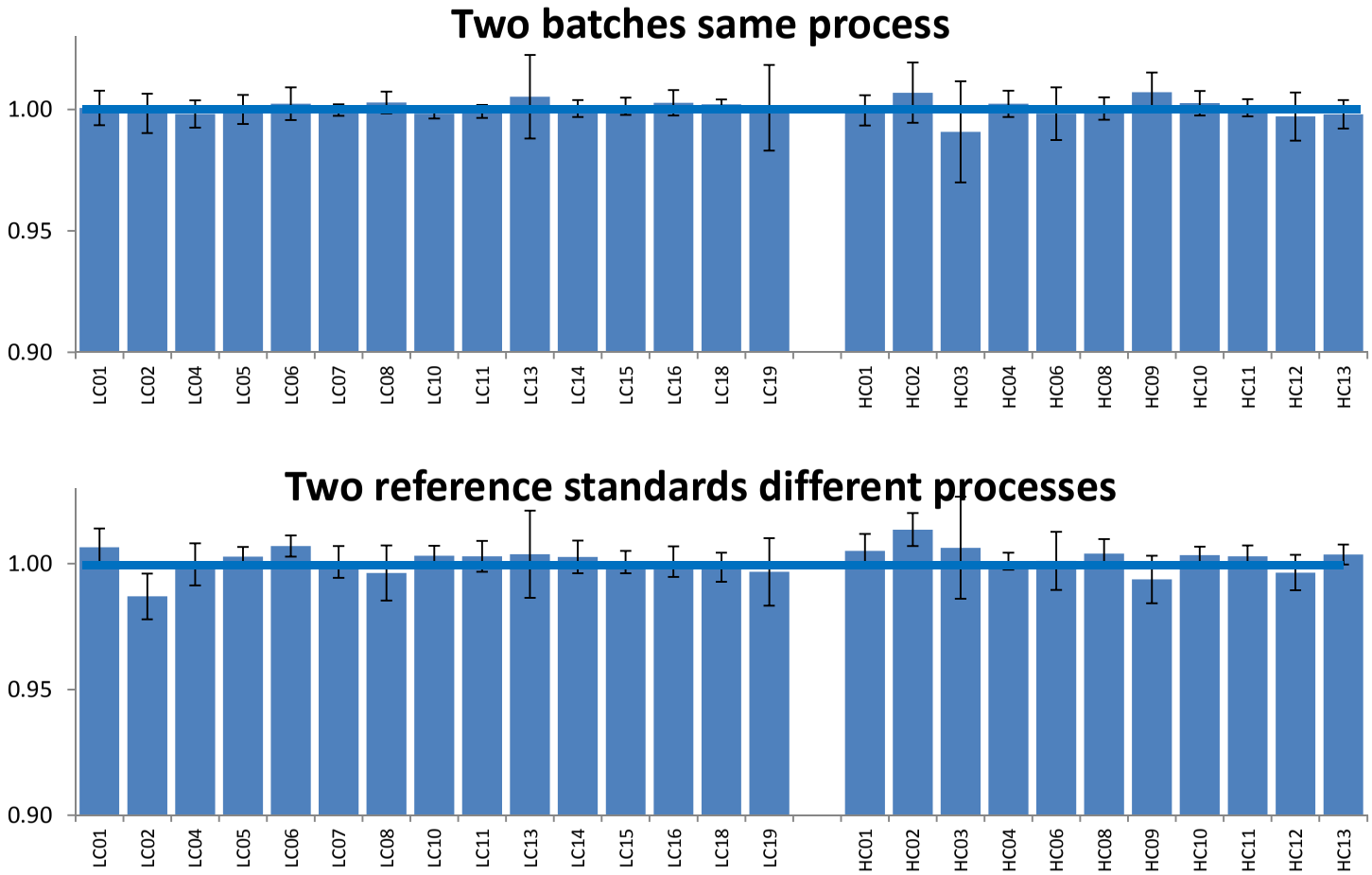
Decrease in signal from peptide results in decrease measured level of peptide

$$\frac{\frac{I_A}{I_B}}{\frac{I_C}{I_D}} = \frac{I_A}{I_C}\frac{I_D}{I_B} = ratio\ of\ sample\ to\ standard$$

Decrease in signal results from modification of the peptide e.g. deamidation, oxidation etc…
…even modifications that we don't know to look for

Labelled internal standard results

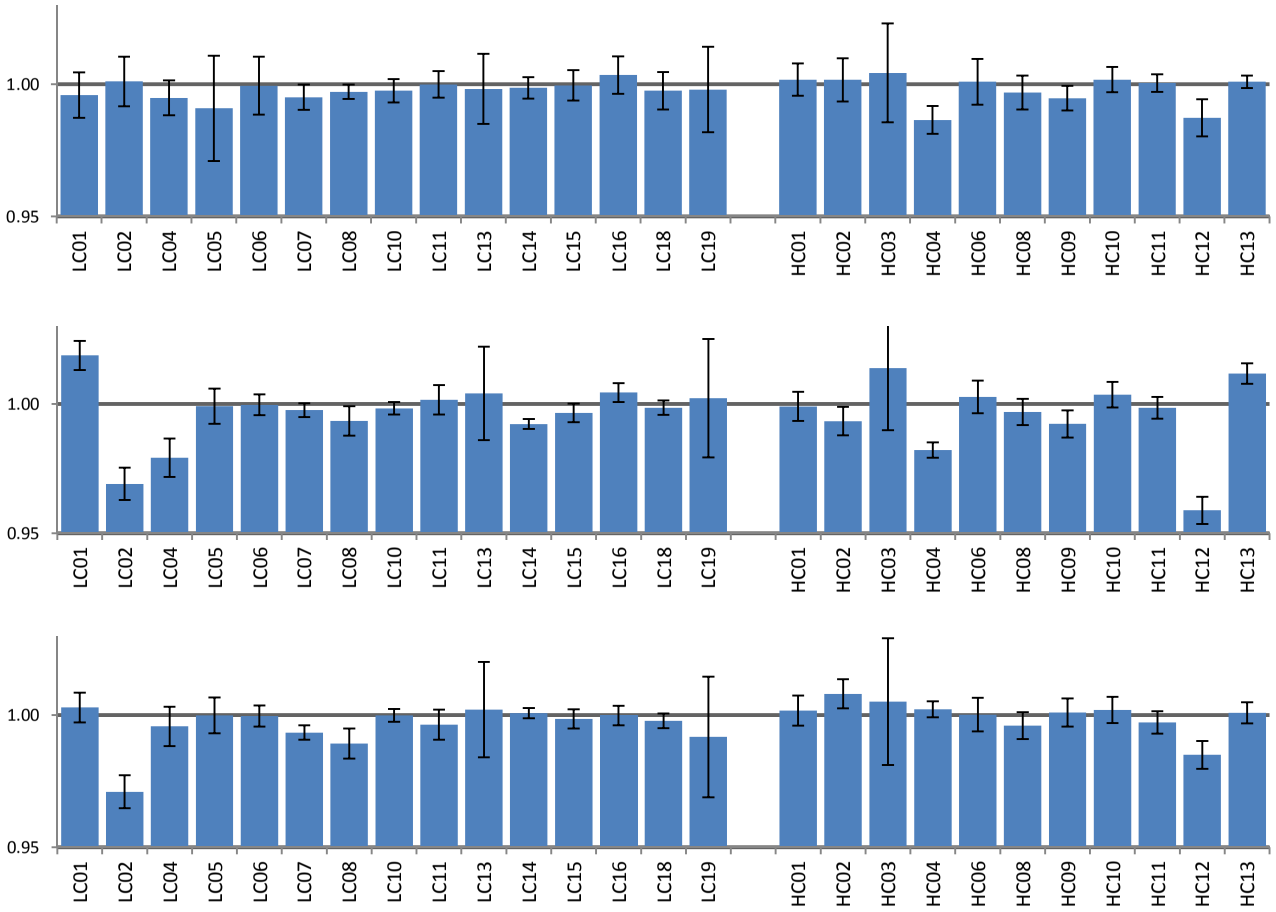
Comparison of similar batches



LC02: 1.3±0.9%
HC02:+1.4±0.7%
0.4% less Wox

Labelled internal standard results

Change of cell line and media



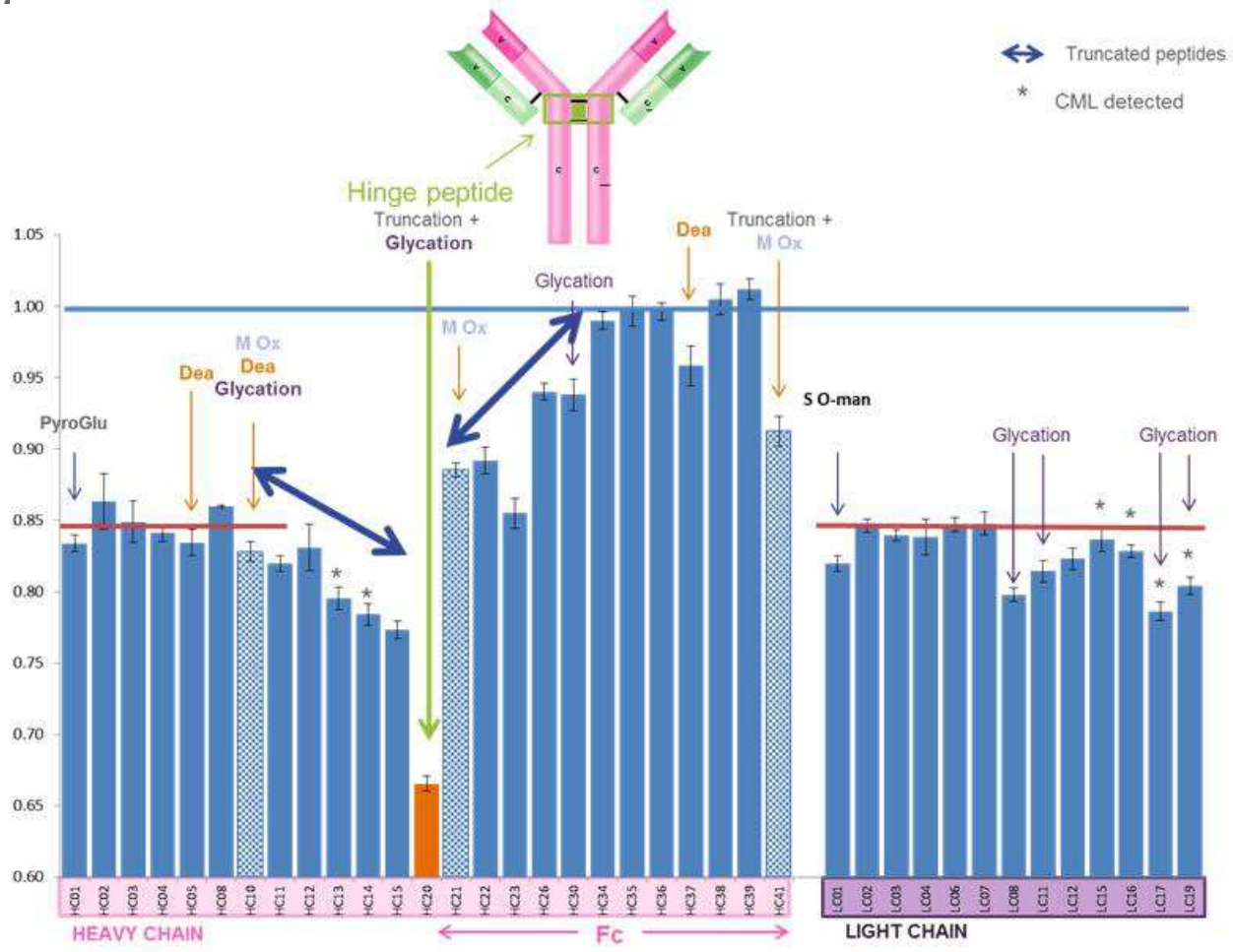
Cell line 1 + Media A

Cell line 2 + Media A

Cell line 1 + Media B

Labelled internal standard results

Acidic species



Statistical methods

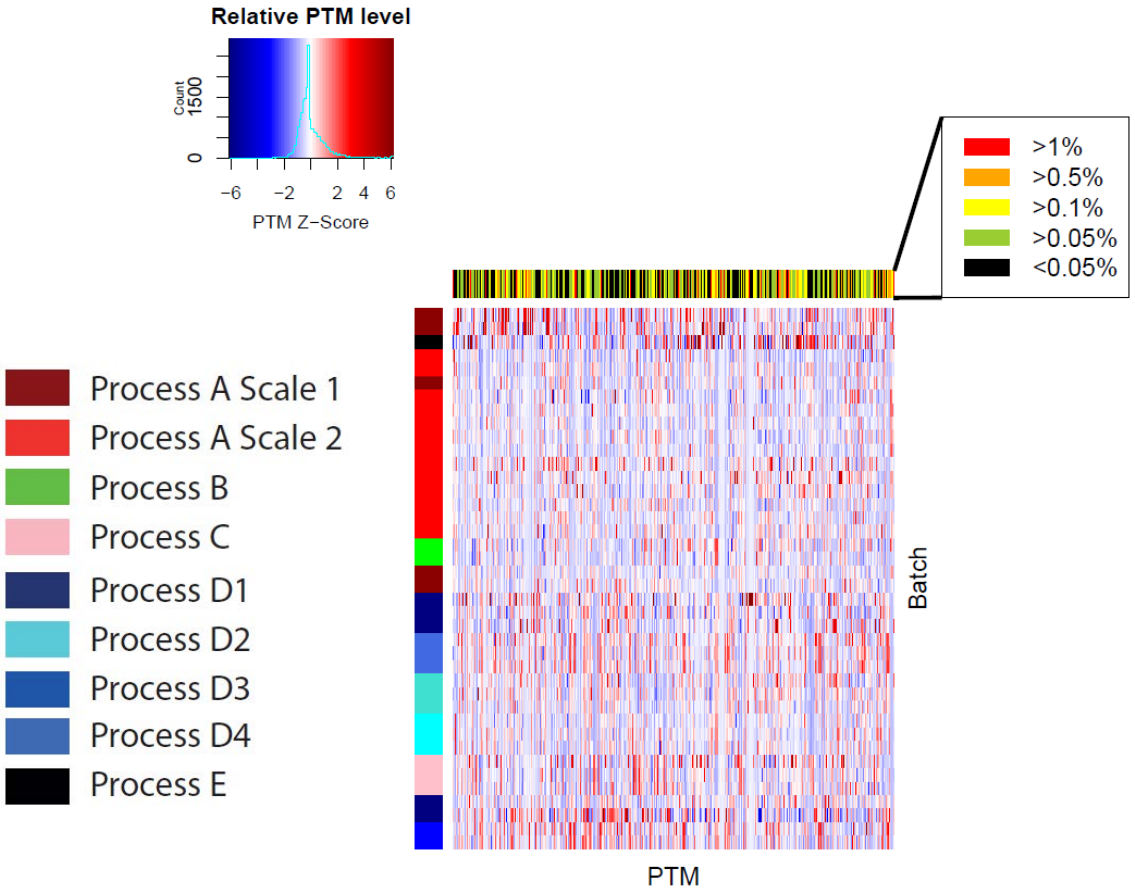
Heatmaps

- Only data used is the levels of PTMs that have been measured for each sample
- Aids the data analysis as using the PTM levels provides some normalisation between the samples
- All modifications are included: hypothesis/non-hypothesis and sequence variants
- For each modification the levels are normalised to the mean and the number of standard deviations from the mean plotted

	Values	Std Dev from mean
Sample 1	0.59	0.71
Sample 2	0.36	-0.11
Sample 3	0.18	-0.75
Sample 4	0.31	-0.29
Sample 5	0.66	0.96
Sample 6	0.19	-0.71
Sample 7	0.06	-1.18
Sample 8	0.87	1.71
Sample 9	0.59	0.71
Sample 10	0.06	-1.18
Mean	0.39	
Std Dev	0.28	

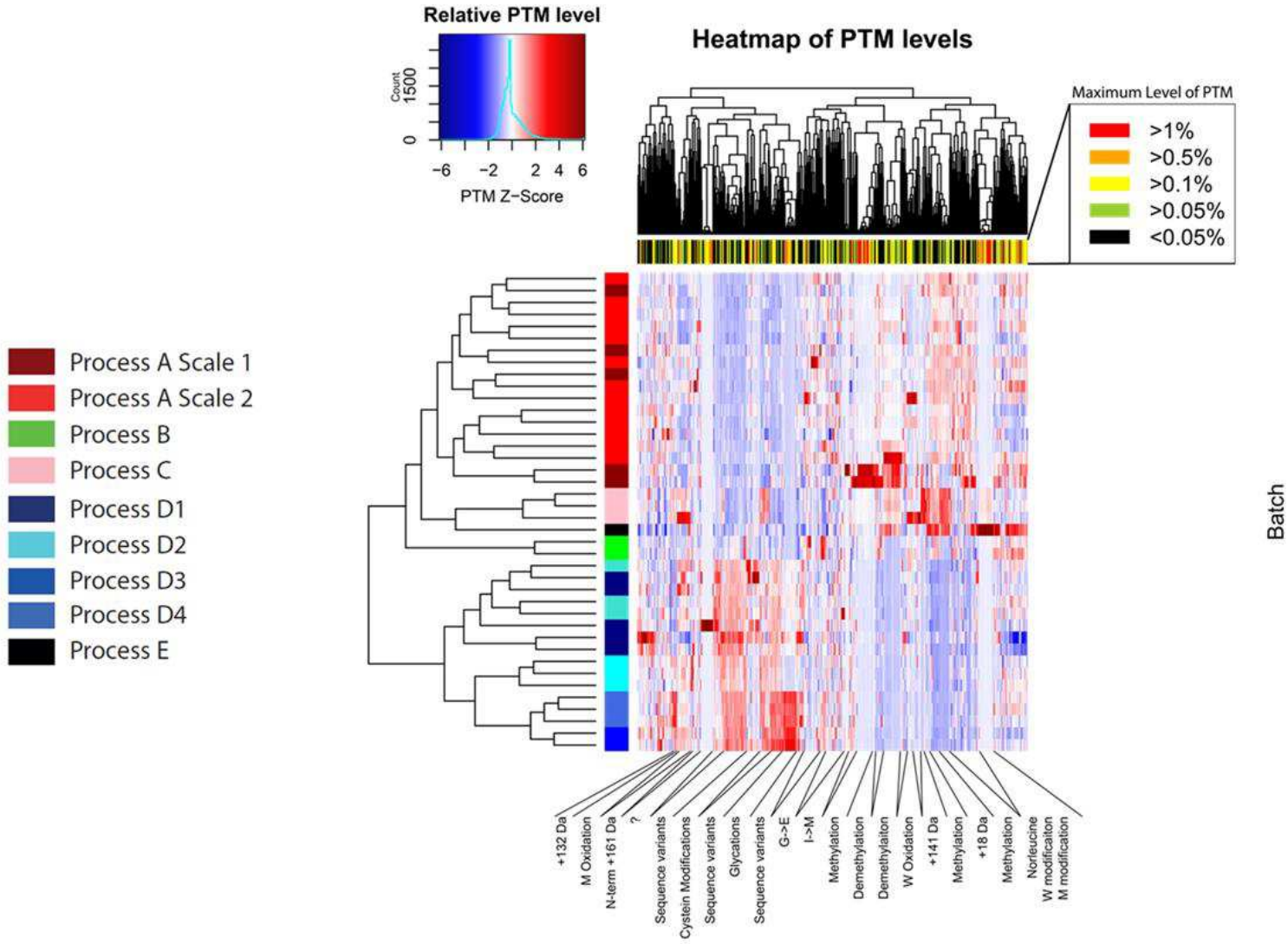
Heatmaps

As originally envisaged…



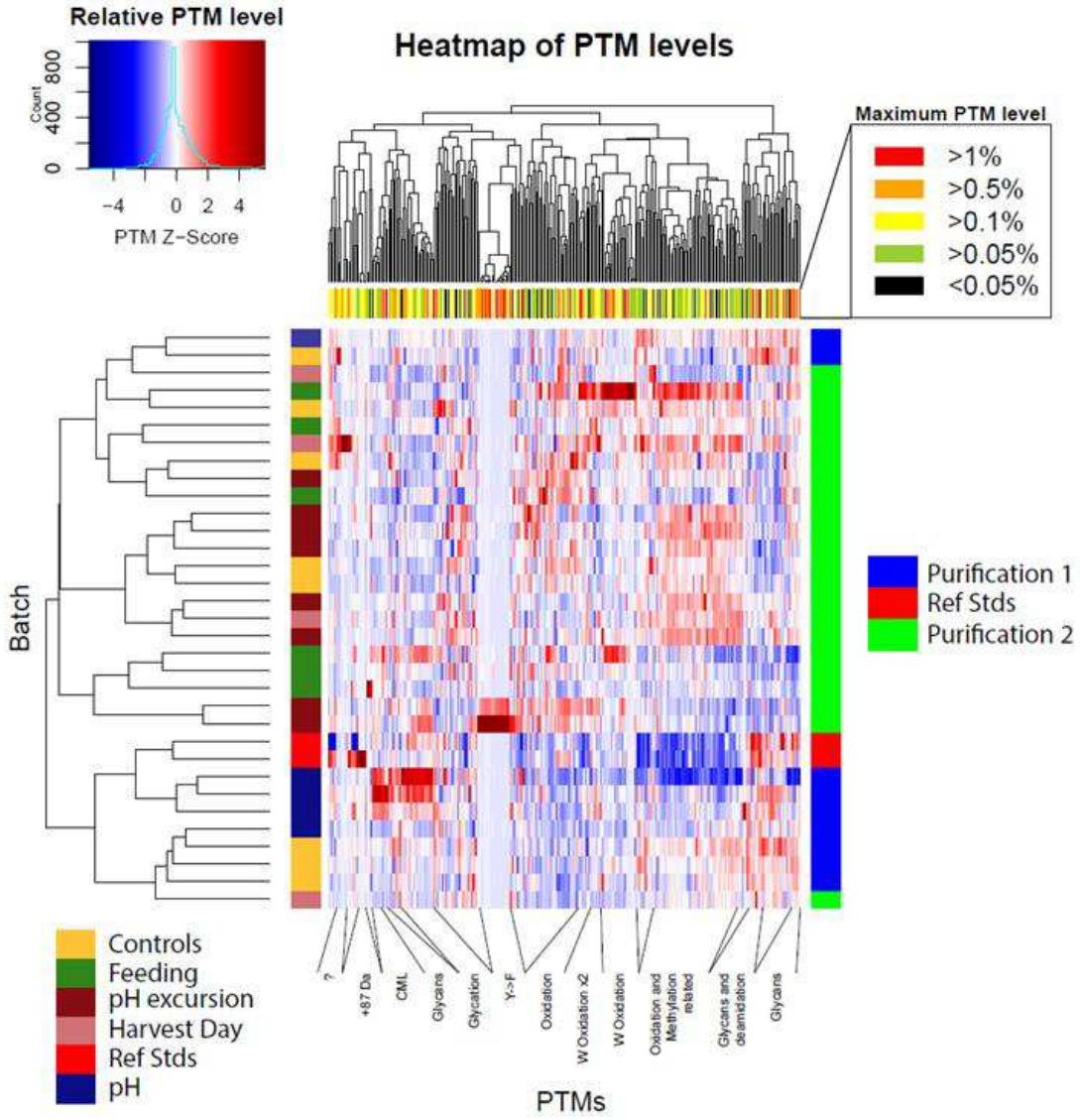
Heat maps

What I got



Heatmaps

Bioreactor conditions



Issues still to be solved

- **Too much data**
- **Combining datasets form multiple occasions**
- **Processing data does not scale well**

Issues still to be solved

Automation

- Current demand requires improvements in automation of sample preparation and data processing

Host Cell Proteins (HCPs)

- Current levels of sensitivity allow detection some of these with our existing methodology

Higher Order Structure

- Can peptide mapping methods be extended to give information on higher-order structure?

Conclusions

- Peptide mapping with mass spectrometry can provide a very detailed analysis of biopharmaceutical samples.
- Large amounts of data are produced.
- Plotting this data as a heat map can aid interpretation of this data and focus attention on potential issues.
- Using isotopically labelled internal standards of the product of interest has the potential to give a rapid comparison of the similarity of batches.
- There is still lots more work to do…

Acknowledgements

Characterisation

Upstream

Questions?

Thanks!