Internal standard approaches in quantification of proteins by LC-MS/MS

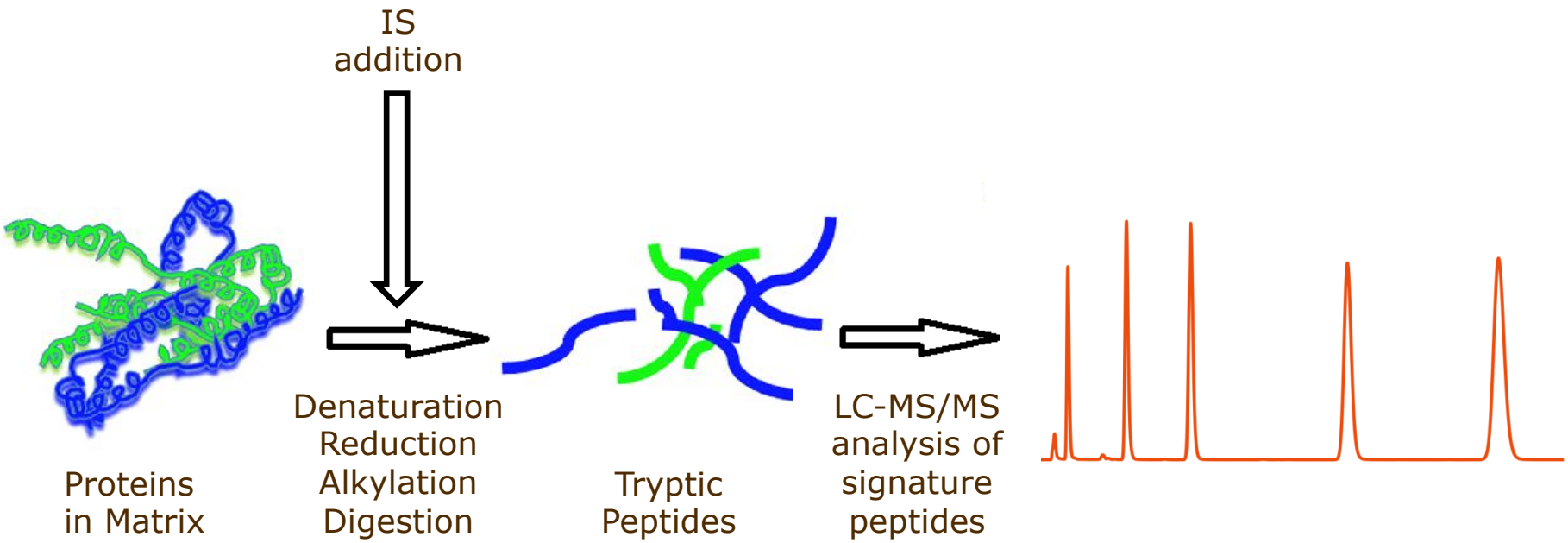
Jérôme and Raffaele, living with epilepsy

Introduction

- Proteins analysis by LC-MS/MS often requires pre-treatment:
  - Reduction
  - Alkylation
  - Digestion

- Internal standard is needed to:
  - Compensate for volumetric variations
  - Compensate for variations in digestion yield
  - Compensate for potential ionization efficiency variations

General Analysis Procedure



Internal Standard Options

- Internal standard options:
  - Isotopically labelled protein
    - Ideal
  - Elongated Isotopically labelled signature peptides
    - **Theoretically** compensates all variations
  - Isotopically labelled signature peptides
    - volumetric and ionization efficiency variations
  - Unrelated proteins
    - Volumetric and digestion yield variations

So when the isotopically labelled protein is not available, what do you chose?

Test Case, methods

- Two methods:

Method 1	Method 2
Fab-PEG Construct	**Monoclonal Antibody**
HPLC method (ACE C18-AR 150x2.1mm, 3µm)	UPLC method (Acquity BEH C18 50x2.1mm, 1.7µm)
ABSciex 5500 Qtrap Shimadzu Nexera	Waters TQS Acquity H-Class

- Selected signature peptide for both compounds is identical, enzymatic digestion is identical as is the sample volume used (10µL)

Test Case, IS approach

- Initial internal standard approach:
  - Elongated (3 amino acids on each side) isotopically labelled signature peptide.

- **Theoretically** compensates for:
  - Volumetric errors
  - Digestion yield
  - Ionization efficiency variations

- Both methods validated using the same internal standard.

Test Case, precision and accuracy results

- Validation results:

	Fab-PEG	Monoclonal
Within Run Inaccuracy	≤ |4.3|%	≤ |10.1|%
Within Run Precision	≤ 4.3%	≤ 4.0%
Between Run Inaccuracy	≤ |4.3|%	≤ |1.7|%
Between Run Precision	≤ 8.6%	≤ 4.5%

Test Case, haemolysed plasma results

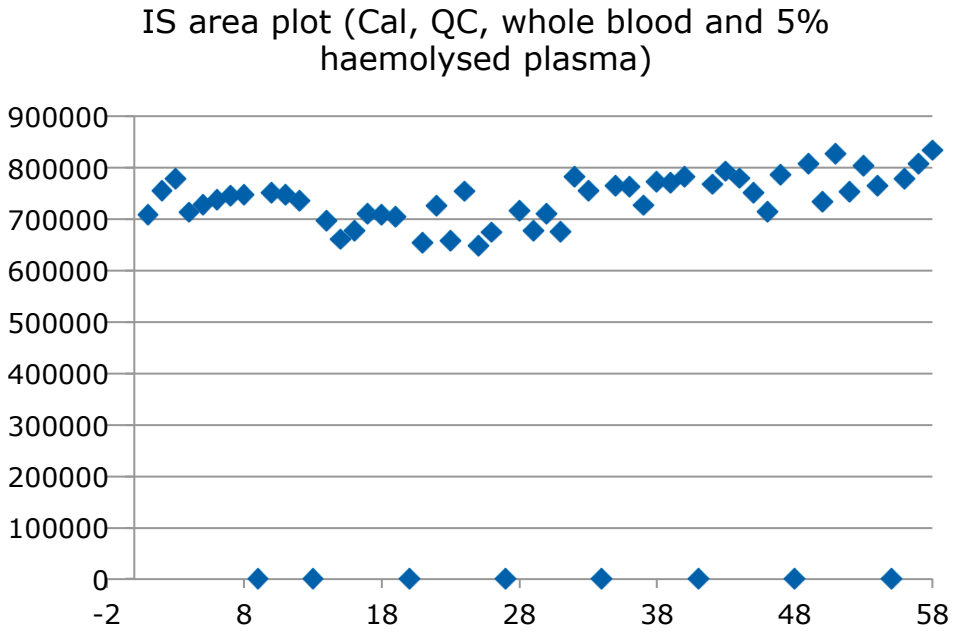
- Heamolysed plasma effect:
  - Whole blood vs. 5% heamolysed blood in plasma

	Fab-PEG			Monoclonal		
	QC Low	QC Mid	QC High	QC Low	QC Mid	QC High
Heamolysed whole blood	**-50.0%**	**-52.1%**	**-53.3%**	**-57.7%**	**-54.3%**	**-53.0%**
5% heamolysed blood in plasma	-11.3%	-11.1%	-14.3%	-17.0%	-8.3%	-7.6%

*results expressed as the deviation (%) of the mean of the observations (n=6) from the nominal concentration.

Test Case, haemolysed plasma results

- 5% haemolysis: more or less OK
- Heamolysed whole blood: large negative deviations.
- IS areas are normal, analyte areas affected by haemolysis.
  ⟹ IS does **NOT** compensate for digestion yield differences



Test Case, haemolysed plasma results

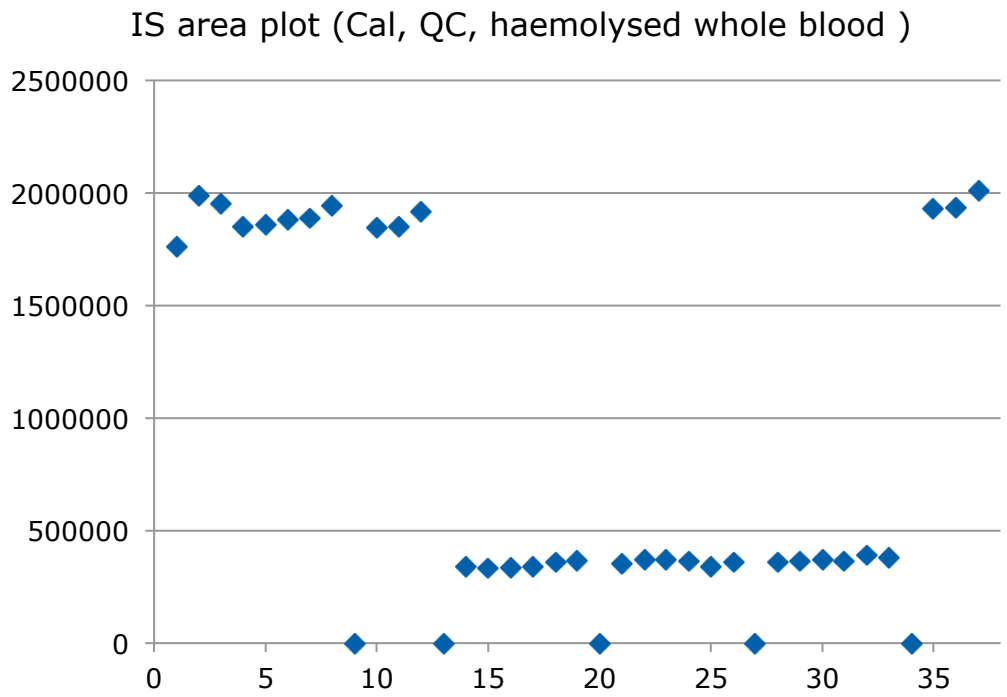
- Large protein as IS:
  - Horse myoglobin

	Fab-PEG			Monoclonal		
	QC Low	QC Mid	QC High	QC Low	QC Mid	QC High
Heamolysed whole blood	**157.2%**	**134.0%**	**132.2%**	3.0%	4.9%	2.9%

*results expressed as the deviation (%) of the mean of the observations (n=6) from the nominal concentration.

Test Case, haemolysed plasma results

- Monoclonal antibody: OK
- Fab-PEG: large positive deviations,
  ⟹ large (larger than for analyte) drop in the IS area.



Test Case, haemolysed plasma results

- Is the IS area drop in the Fab-PEG test an ionization effect on the ABSciex 5500?

- Reinjection of samples using the monoclonal antibody method (Waters TQS)

	Fab-PEG		
	QC Low	QC Mid	QC High
Heamolysed whole blood	8.1%	6.6%	3.8%

*results expressed as the deviation (%) of the mean of the observations (n=6) from the nominal concentration.

- Fab-PEG results OK on the TQS

  ⟹ ionization problem on the ABSciex 5500

Test Case, what causes the drop in analyte signal

- Increased protein content causes signal drop?
  - Test in plasma with 0.4g/mL of BSA added.

	Fab-PEG		
	QC Low	QC Mid	QC High
Plasma + 0.4g/mL BSA	**-48.5%**	**-51.2%**	**-50.3%**

*results expressed as the deviation (%) of the mean of the observations (n=6) from the nominal concentration.

Question

- Assuming an isotopically labelled protein is not available, can we use an internal standardisation system which would allow to overcome all issues associated with these analysis?

Hypothesis

- Use of three internal standard:
  - The elongated isotopically labelled analyte signature peptide (IS1)
  - Horse myoglobin (IS2)
  - The elongated isotopically labelled myoglobin signature peptide (IS3)

- The response becomes:

$$Response = Analyte\ area / IS1 / IS2 / IS3$$

This approach should eliminate all sources of variability

Test Case, three IS approach

- Test in haemolysed whole blood with three internal standards:

	Fab-PEG			Monoclonal		
	QC Low	QC Mid	QC High	QC Low	QC Mid	QC High
Heamolysed whole blood	12.6%	7.7%	0.7%	7.4%	7.5%	5.5%

*results expressed as the deviation (%) of the mean of the observations (n=6) from the nominal concentration.

- All results acceptable

  ⟹ compensation of both ionization efficiency and digestion yield

Conclusion

- Internal standardization for bioanalysis of proteins by MS remains a challenge since very often the isotopically labeled protein is not available
- The different sources of variability (volumetric, digestion yield, ionization yield) can be eliminated by using a multiple internal standard approach
- The applicability of the system for routine use remains challenging, especially from a software point of view and especially in a regulated environment

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Questions?