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Design of Experiment

A Powerful Tool to Optimise Sample Preparation in Bottom-up Targeted Protein LC-MS Workflows

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Drug Development Solutions

15th EBF Open Symposium
17th November 2022 Barcelona

Conventional Optimisation

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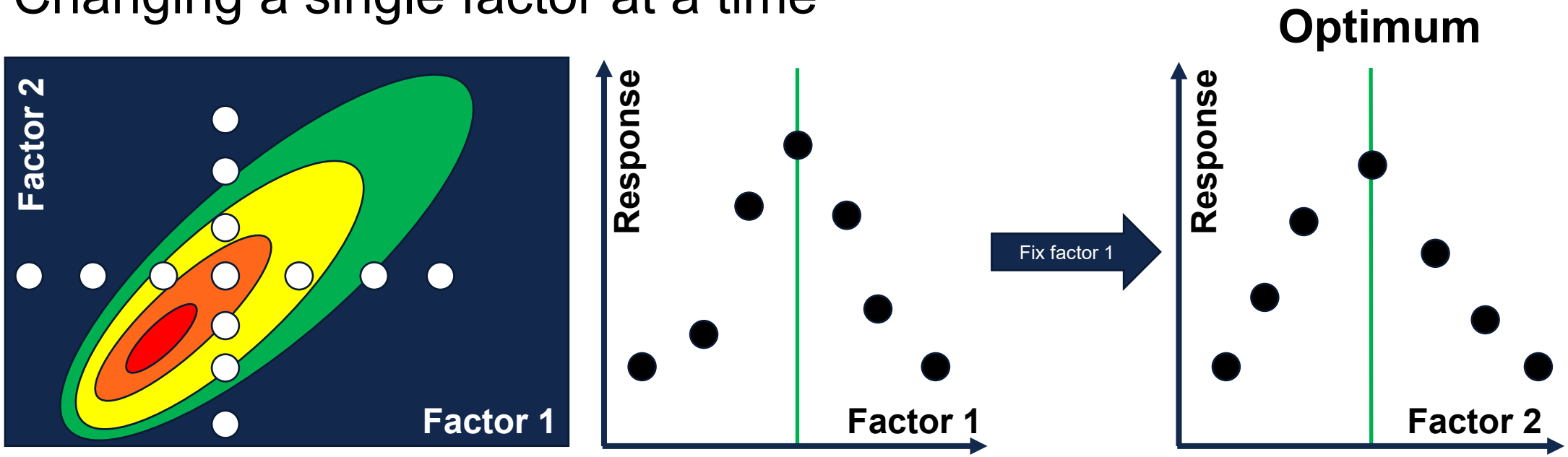
Changing a single factor at a time



- Does not always lead to real optimum
- Limited information
- Many experiments

Design of Experiment (DoE)

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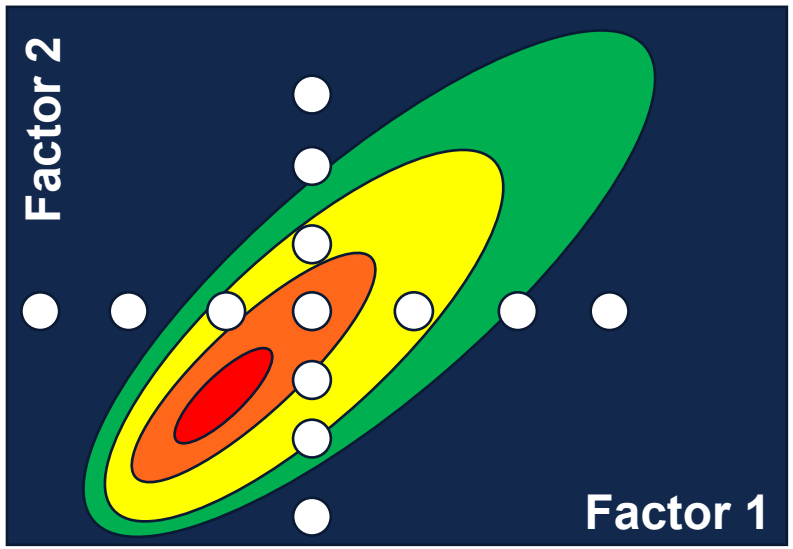
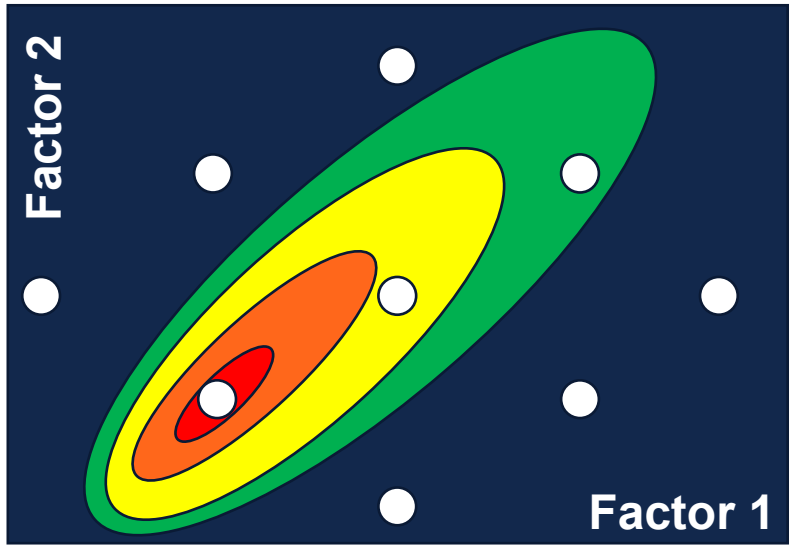
Conventional



VS

DoE



- A strategically planed and executed series of experiments
- All factors (e.g. pH, solvent, temperature) are changed simultaneously
- Allows to investigate multiple factors at the same time
- More information, model setup and predictive power
- Fewer experiments

Protein LC-MS Quantitation

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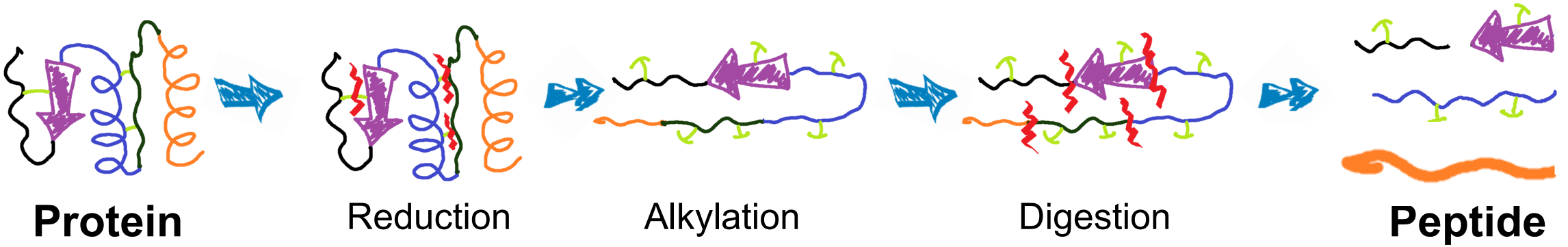
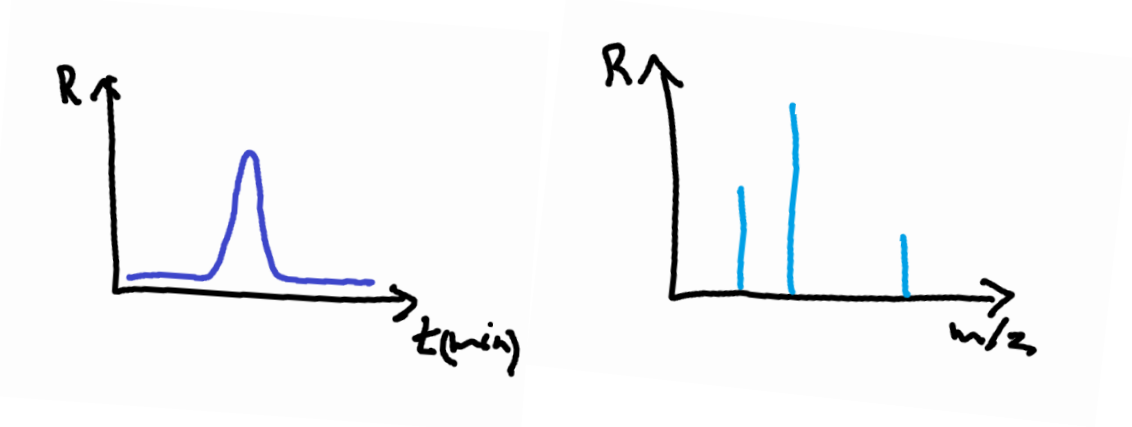
Bottom-up approach



- Unique peptide selected
- Peptide analysis by LC-MS/MS



Problem

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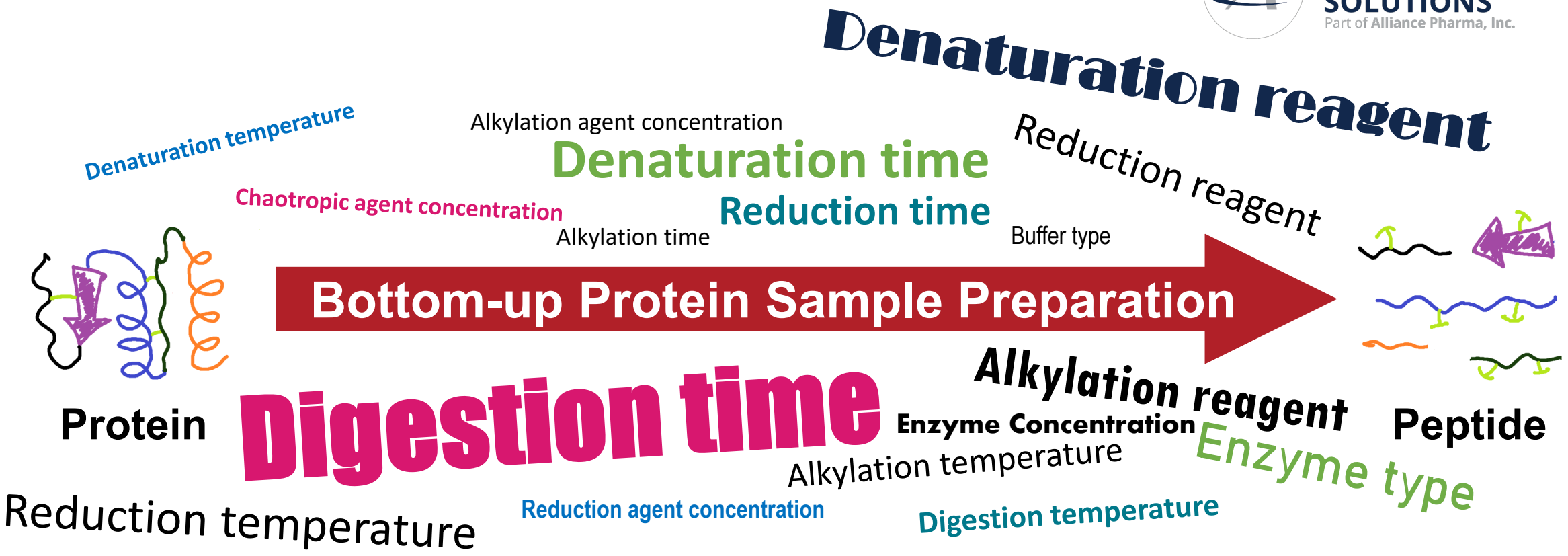

- 17 variables @ 2 levels at all combinations → 2^{17} = ~130,000 experiments
- Full optimisation is not attempted
- Generic methods (empirical, historical) – “Worked fine before”
- **DoE for the help**

DoE Workflow

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1. Define objectives
2. Define factors
3. Selection of experimental design
4. Perform experiment
5. Process the data

DoE Workflow

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- Model analyte: IgG1 antibody
- Spiked into rat plasma
- 4 abundant HC surrogate peptides selected:

  DTLM
  FNWY
  TTPV
  VVSV

Goal

- Maximize the response for the 4 surrogate peptides selected

DoE Workflow

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What variable do we want to assess? At what levels?

- Reaction buffer ✗
- Chaotropic agent: guanidine, urea
- Reduction agent: DTT, TCEP
- Reduction agent concentration: 1 - 50 mM
- Reduction incubation time: 10 - 60 min
- Reduction incubation temperature: 22 - 70°C
- Alkylation conditions ✗
- Protease enzyme type: methylated, non-methylated trypsin
- Enzyme to protein ratio (amount of enzyme): 1:5 – 1:500
- Digestion time: 1.5 hours - O/N

DoE Workflow

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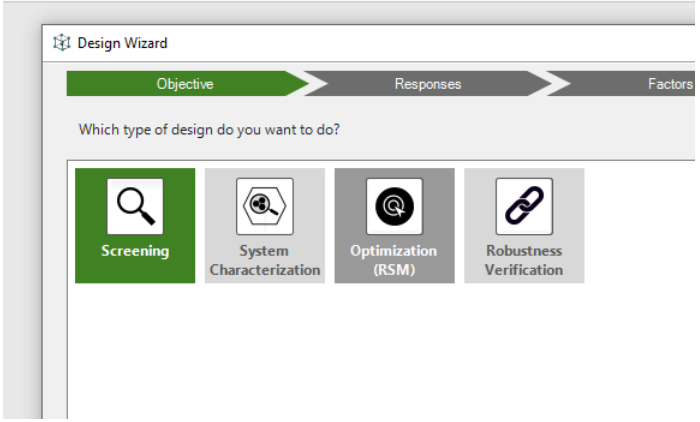
Modde Go software package



- Design Wizard
- **Screening**
  - Test a large number of factors
  - Normally 2 or 3 levels
  - What factors have the most impact on the assay?
- **Optimisation**
  - Smaller number of factors
  - Min. 3 levels
  - Model generation, prediction
  - Find the best conditions
- **Output:** experiment table

	1	2	3	4	5	6	7	8	9	10
	Exp No	Exp Name	Run Order	Incl/Excl	Enzyme	Chaotropic Agent	Reduction Agent	Reduction Agent Concentration	E/P ratio	reduction Temperature
1	1	N1	1	Incl	Promega	Guanidine	DTT	5	50	22
2	2	N2	5	Incl	Thermo	Guanidine	DTT	5	100	22
3	13	N13	17	Incl	Promega	Guanidine	TCEP	50	100	22
4	14	N14	14	Incl	Thermo	Guanidine	TCEP	50	50	22
5	11	N11	9	Incl	Promega	Urea	DTT	50	100	22
6	12	N12	13	Incl	Thermo	Urea	DTT	50	50	22
7	7	N7	4	Incl	Promega	Urea	TCEP	5	50	22
8	8	N8	2	Incl	Thermo	Urea	TCEP	5	100	22
9	17	N17	3	Incl	Thermo	Urea	DTT	27.5	75	46
10	18	N18	19	Incl	Thermo	Urea	DTT	27.5	75	46
11	19	N19	22	Incl	Thermo	Urea	DTT	27.5	75	46
12	20	N20	20	Incl	Promega	Urea	DTT	27.5	75	46
13	21	N21	21	Incl	Promega	Urea	DTT	27.5	75	46
14	22	N22	18	Incl	Promega	Urea	DTT	27.5	75	46
15	9	N9	15	Incl	Promega	Guanidine	DTT	50	50	70
16	10	N10	10	Incl	Thermo	Guanidine	DTT	50	100	70
17	5	N5	12	Incl	Promega	Guanidine	TCEP	5	100	70
18	6	N6	7	Incl	Thermo	Guanidine	TCEP	5	50	70
19	3	N3	16	Incl	Promega	Urea	DTT	5	100	70
20	4	N4	6	Incl	Thermo	Urea	DTT	5	50	70
21	15	N15	8	Incl	Promega	Urea	TCEP	50	50	70
22	16	N16	11	Incl	Thermo	Urea	TCEP	50	100	70

DoE Workflow

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1. Define objectives
2. Define factors
3. Selection of experimental design
4. Perform experiment
5. Process the data

Perform experiments

- Following experiment table
- Samples injected in a random order

- Waters Acquity Classic UPLC
  - Acquity UPLC peptide CSH C18 2.1x100 mm, 130 Å, 1.7 µm

- Waters Xevo TQS
  - Triple quadrupole

DoE Workflow

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1. Define objectives
2. Define factors
3. Selection of experimental design
4. Perform experiment
5. Process the data

Modde Go software package

- Responses (peak area) imported from LC-MS data processing software
- Interpretation of results
- Visualisation
- Modelling
- Prediction of optimal conditions

Screening Design

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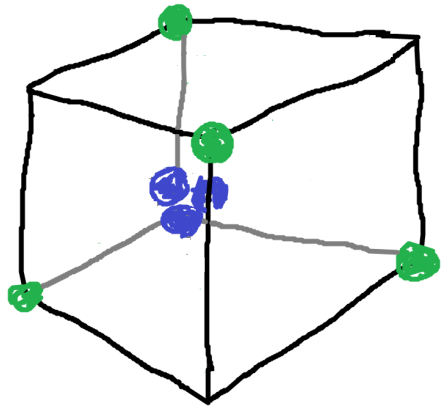
- 6 factors at 2 or 3 levels
- Digestion time not included in the design performed twice: 1.5 hr and O/N digestion
- 3 replicates at center-point to assess variation



- **Fractional factorial design**
- **44** samples (injections)

Factor	Levels
Chaotropic agent	Guanidine Urea
Reduction reagent	DTT TCEP
Trypsin	Methylated Non-methylated
Reduction agent concentration (mM)	5 27.5 50
Reduction temperature (°C)	22 46 70
E/P ratio	1:50 1:75 1:100

Screening design – Results

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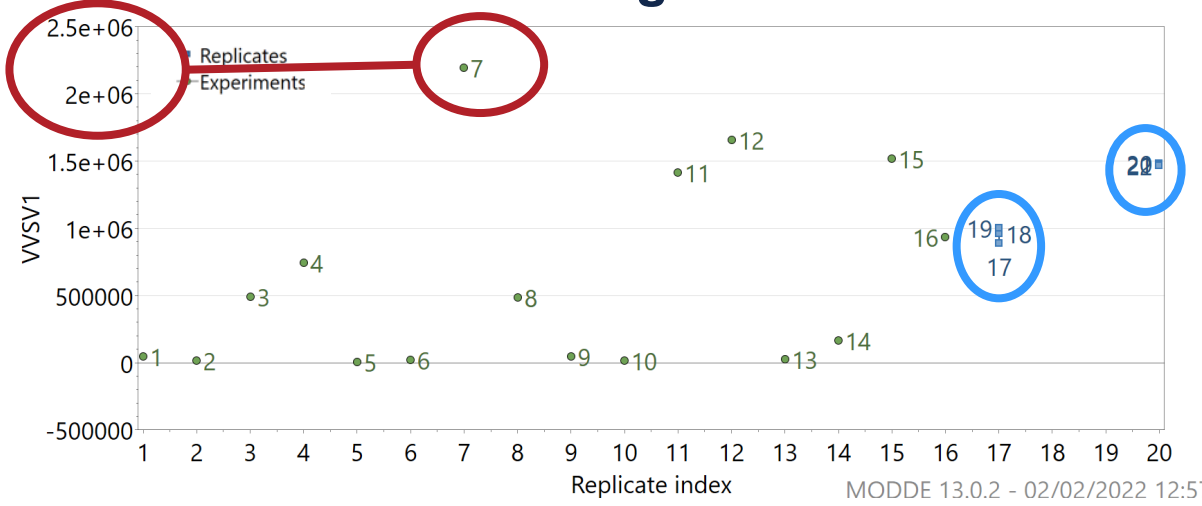
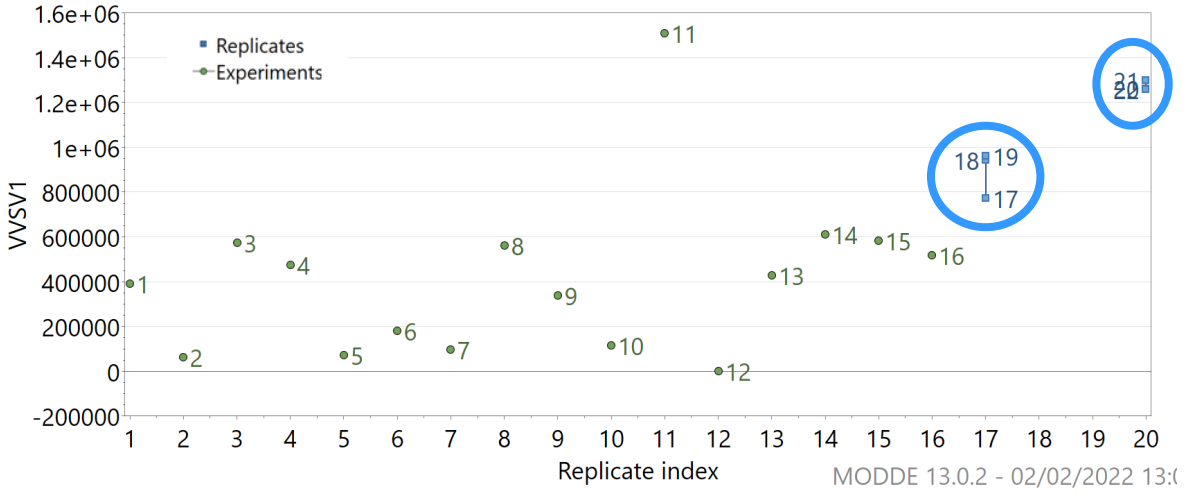
Replicate plots for VVSV surrogate peptide

1.5-hour digestion



O/N digestion



- Center-point replicates (**blue squares**) are very tight → **high data quality**
- Highest response is obtained by short digestion → **O/N digestion not required**

Screening design - Results

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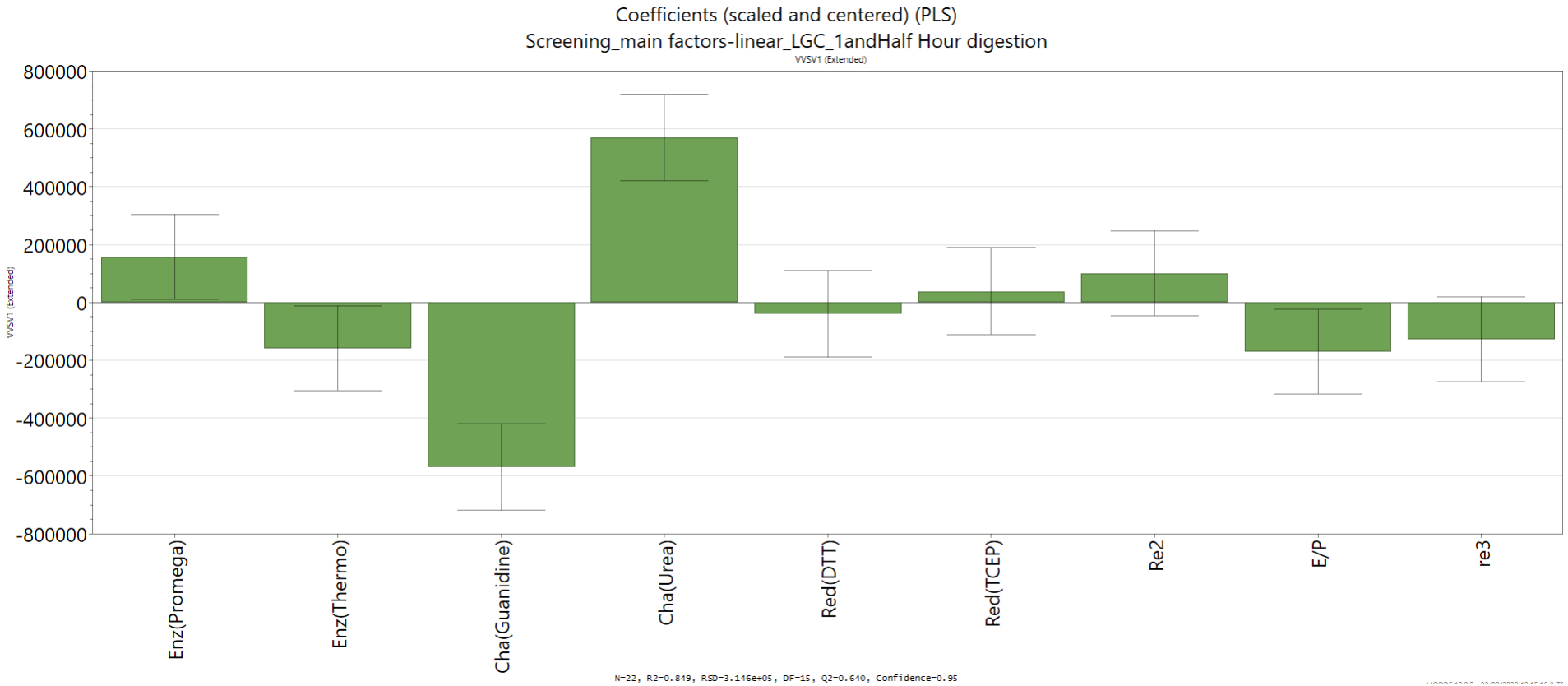
What is significant?

Coefficient plot for VVSV surrogate peptide



Conclusion

Urea/DTT fixed
Decrease E/P

Next step

Optimisation design

Optimisation Design

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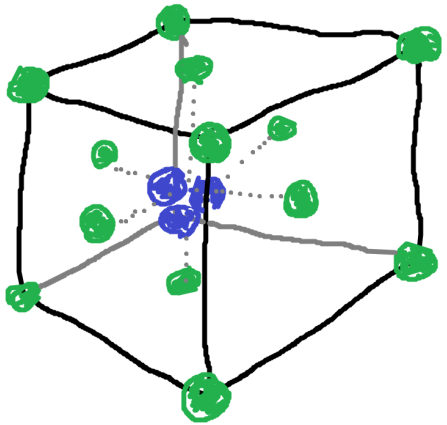
- 4 factors at 3 levels
- Methylated trypsin not affordable at low E/P
- 3 replicates at center-point



- **Reduced central composite face centered design**
- **23** samples (injections)

Factor	Levels
DTT concentration (mM)	1 13 25
Reduction temperature (°C)	22 46 70
Reduction time (min)	10 35 60
E/P ratio	5 27.5 50

Optimisation – Results

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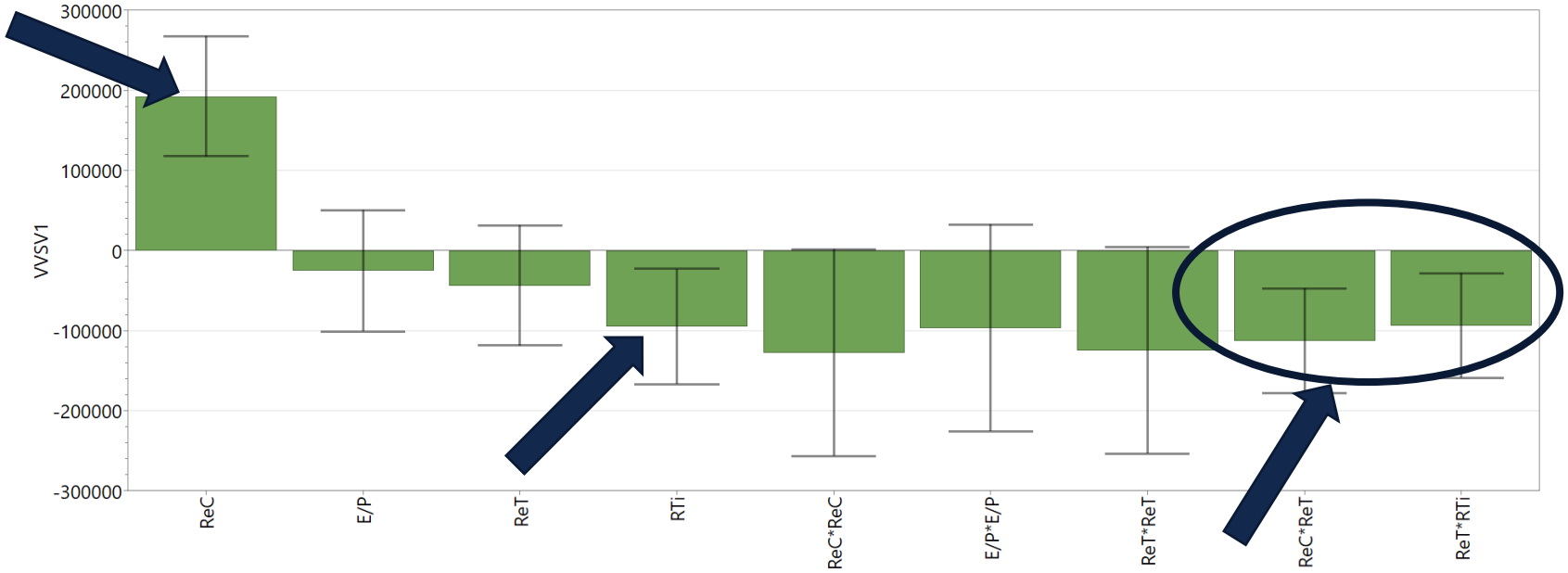
Coefficient plot for VVSV surrogate peptide



- DTT concentration improves the response
- Reduction time has a negative effect
- Interaction effects detected: DDT conc. x temperature and temperature x time

Optimisation – Results

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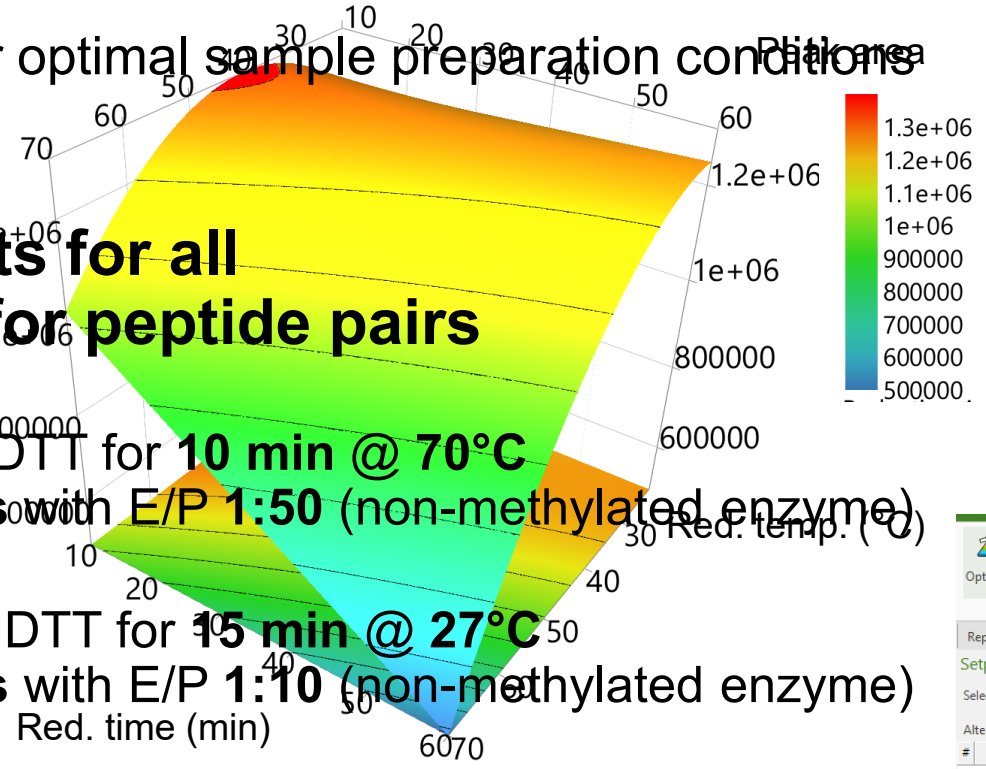
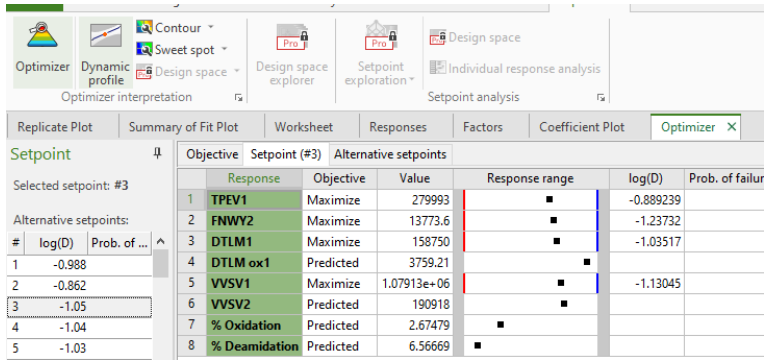
- Model generated
- Prediction
  - **Modde Optimizer** for optimal sample preparation conditions

Surface response plot for VVSV



Outcome

- **No single method fits for all**
- **2 optimal methods for peptide pairs**
  1. DTLM & FNWY:
     - Reduce with **1 mM** DTT for **10 min @ 70°C**
     - Digest for **1.5 hours** with E/P **1:50** (non-methylated enzyme)
  2. TPEV & VVSV:
     - Reduce with **23mM** DTT for **15 min @ 27°C**
     - Digest for **1.5 hours** with E/P **1:10** (non-methylated enzyme)



Next step: assess optimal methods vs a generic preparation

Optimisation – Prediction Validation

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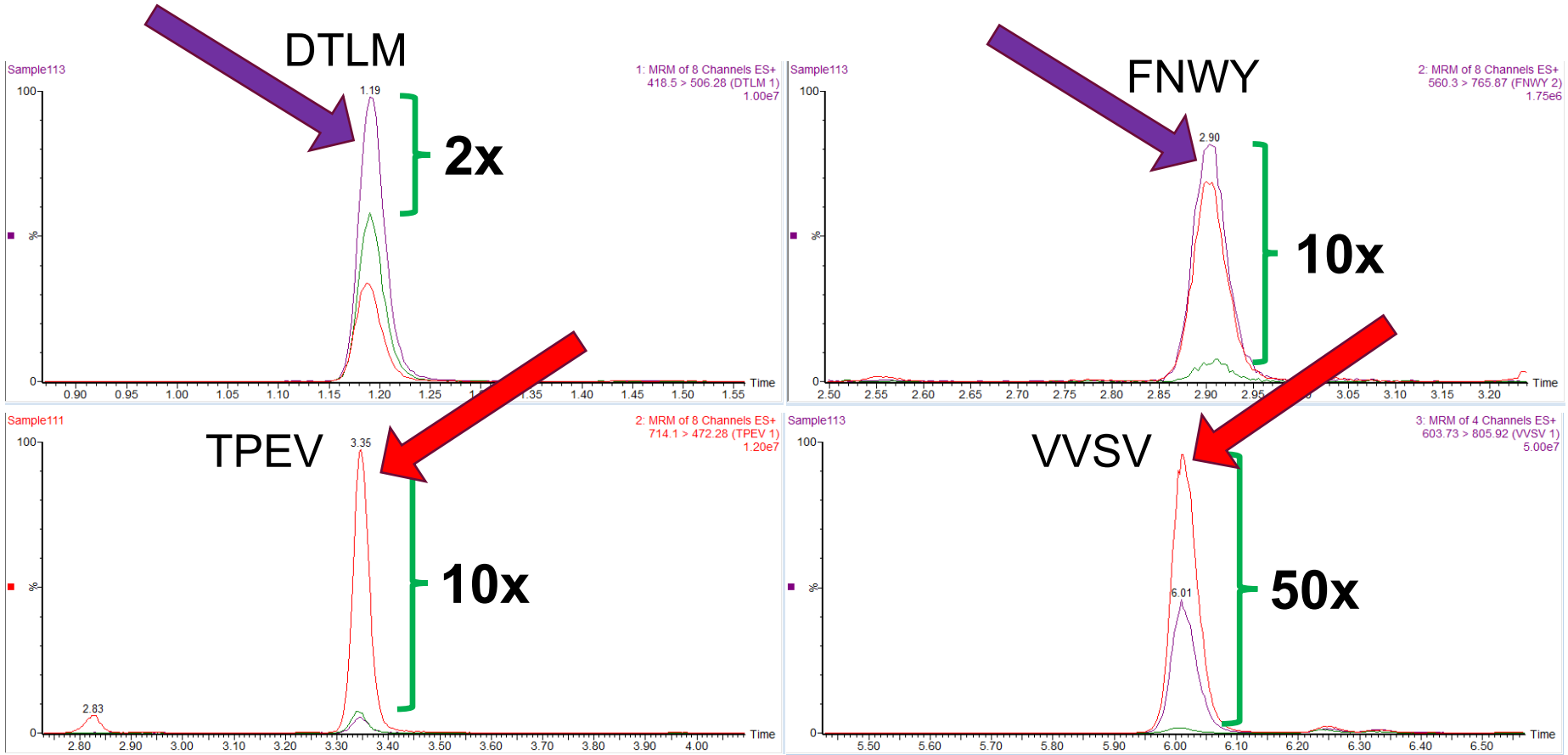

- Optimal preparation conditions for **DTLM** and **FNWY** by **DoE** (~3-hour prep.)
- Optimal preparation conditions for **TPEV** and **VVSV** by **DoE** (~3-hour prep.)
- **Control** generic preparation setup (2-day prep.)

Conclusions

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- DoE excellent tool for protein sample preparation optimisation for LC-MS assays
- Achieved comprehensive optimisation within minimal experiments (~70 vs ~500)
- Reliable predictive power – responses changed as predicted by the model
- Peptide yields from IgG1 increased by **10-50x** → **increased sensitivity**
- Significant reduction of sample preparation time (**~3 hours** vs O/N)

  → **higher throughput**
- Challenge: difficult to execute in the lab → looking at automation options

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Special Thanks!

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Professor

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Thank you for your attention

Any further questions?

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