FACULTY OF
PHARMACEUTICAL SCIENCES

DEPARTMENT OF BIOANALYSIS – LABORATORY OF TOXICOLOGY

THE SIMULTANEOUS QUANTIFICATION OF THIAMINE, ITS PHOSPHATE DERIVATIVES AND PRECURSORS IN *ARABIDOPSIS*

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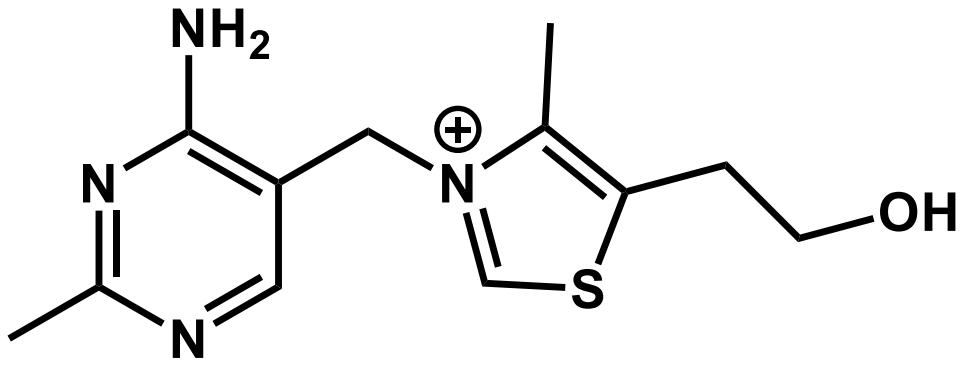
INTRODUCTION

- Vitamin B1
- Energy metabolism
- Deficiency > Beri-Beri
- Low content in major food crops, e.g. rice
- Mainly problem in developing countries



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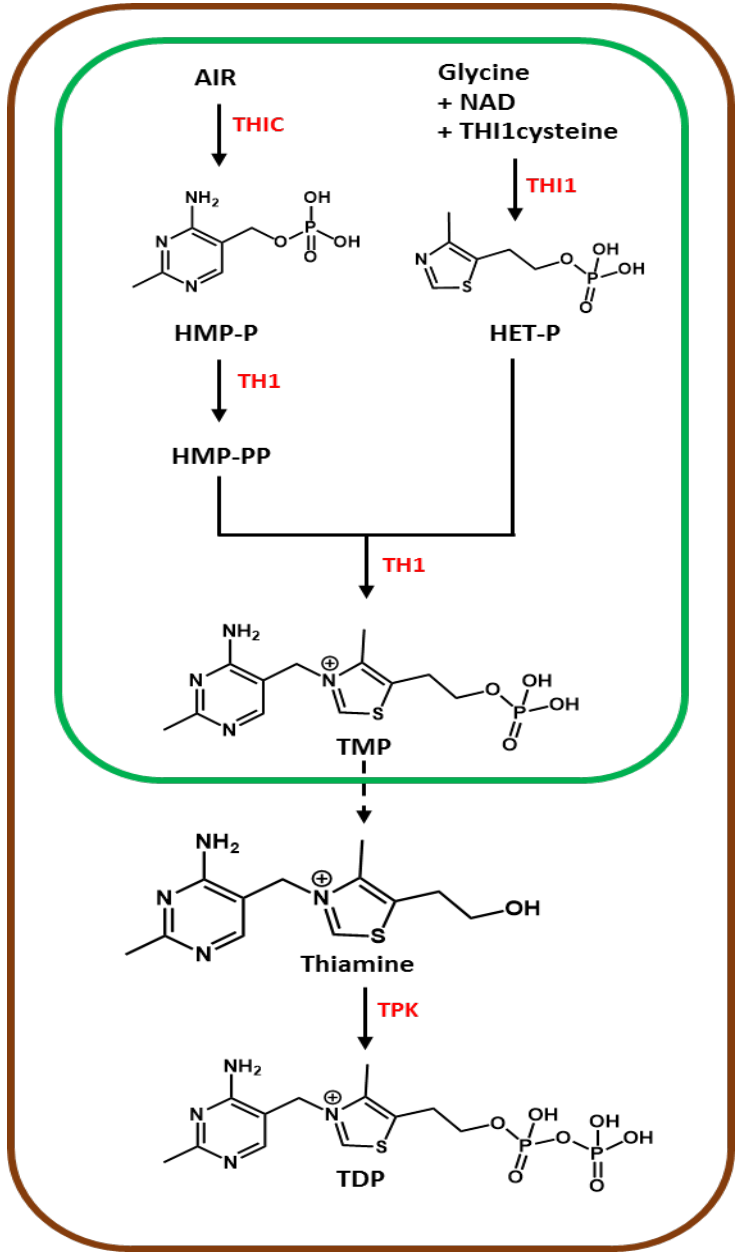
INTRODUCTION

- ↑ thiamine levels via genetic modification of key enzymes in thiamine bio-synthesis



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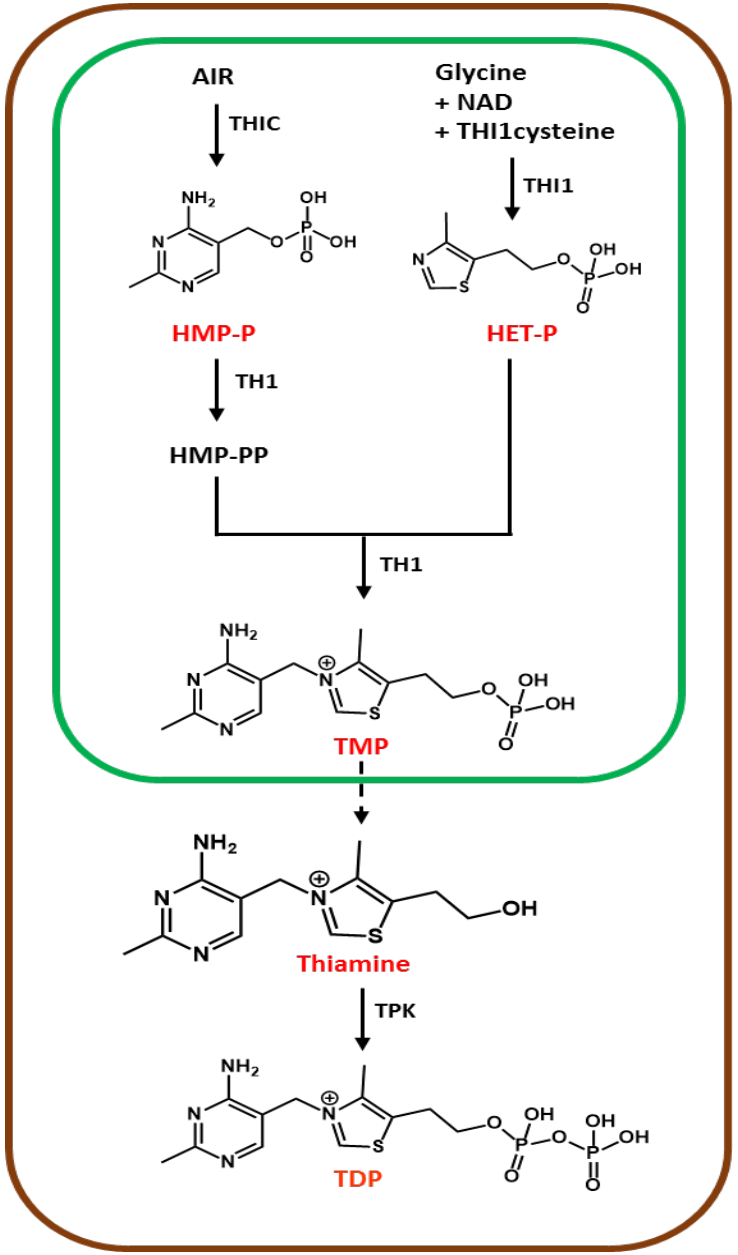
INTRODUCTION

HOW?

- Need for knowledge about regulation of thiamine bio-synthesis in plants

→ determination direct products of key enzymes

- *Arabidopsis thaliana* = reference plant



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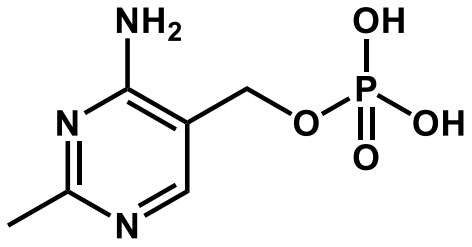
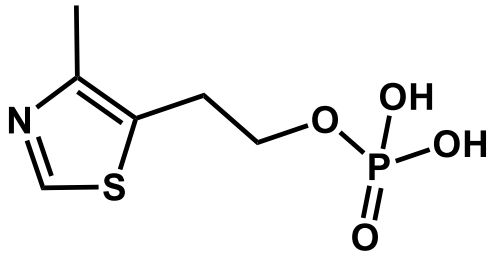
AIM OF THE STUDY

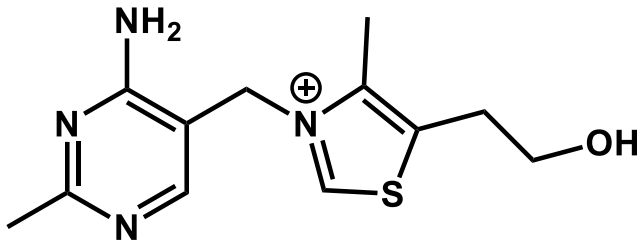
DEVELOPMENT OF LC-MS/MS METHOD FOR THE DETERMINATION OF THIAMINE, ITS PHOSPHATE DERIVATIVES AND PRECURSORS IN *ARABIDOPSIS THALIANA*



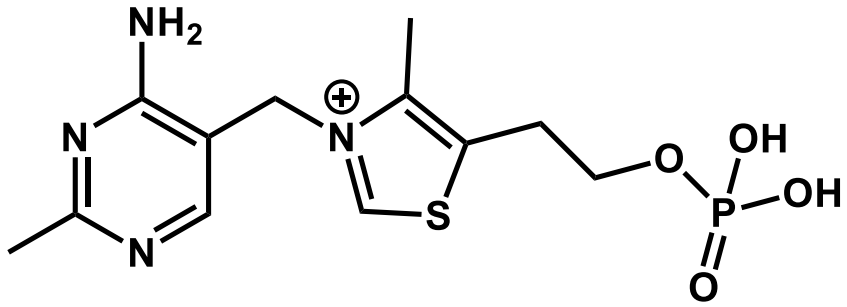
HMP-P



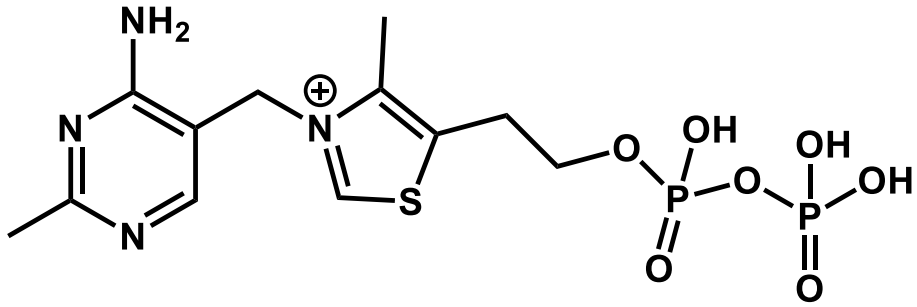
HET-P



Thiamine



Thiamine monophosphate (TMP)



Thiamine diphosphate (TDP)

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CHALLENGES

- Polar compounds
- Ionogenic compounds
- Chelating compounds

→ **Chromatography**

- Labile compounds

→ **Sample preparation**

- Endogenous compounds

→ **Validation**

- No reference standards of HMP-P and HET-P
- No labeled internal standards for all compounds

→ **Quantification**

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OPTIMISATION OF THE CHROMATOGRAPHY

Phenomenex Gemini NX C18

Mobile phase A: 10 mM NH_4CO_3 pH 8.8

Mobile phase B: MeOH

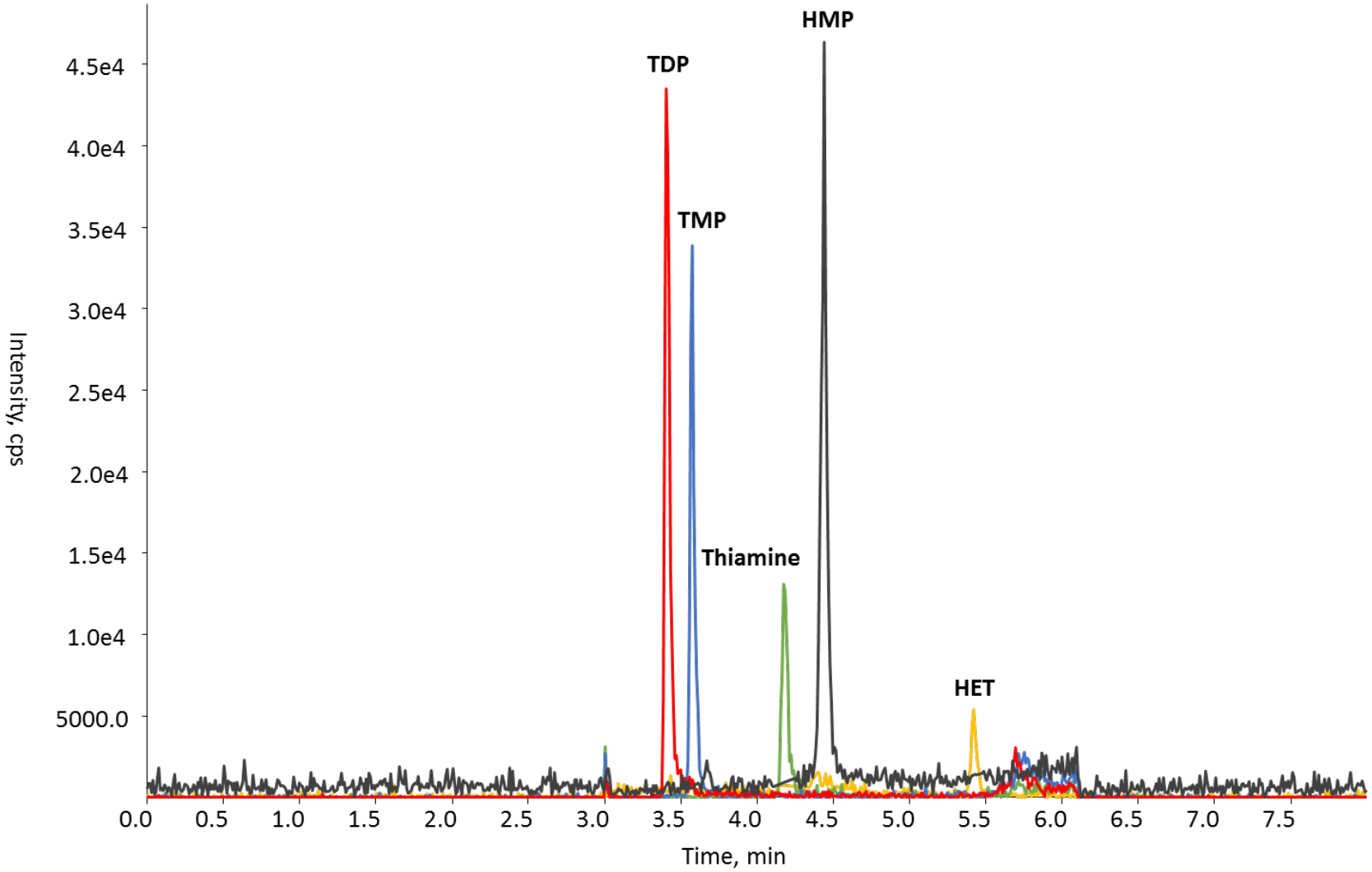
8-min gradient run

<u>LLOQ level</u>:

TDP, 7 ng/ml; TMP, 0.9 ng/ml;

thiamine, 3.6 µg/ml; HMP, 0.17 ng/ml;

HET, 0.09 ng/ml



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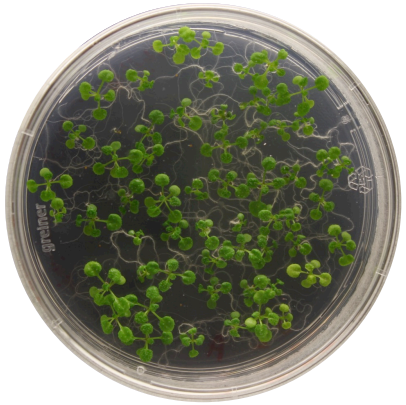
OPTIMISATION OF THE SAMPLE PREPARATION PROCEDURE



200 mg

+ 1.5 ml 0.1 M HCl

30’ at 74°C

10’ at 30 Hz

+ 10U phosphatase → **Total thiamine:** thiamine + TDP + TMP
Total HET: HET + HET-P
Total HMP: HMP + HMP-P + HMP-PP → 24h at 45°C

- phosphatase → **TDP, TMP, thiamine, HET and HMP** → 24h at 4°C

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VALIDATION

Parameters to evaluate:

- Selectivity
- Carry-over
- Calibration model
- Accuracy and precision
- Dilution integrity
- Matrix effect
- Recovery
- Stability

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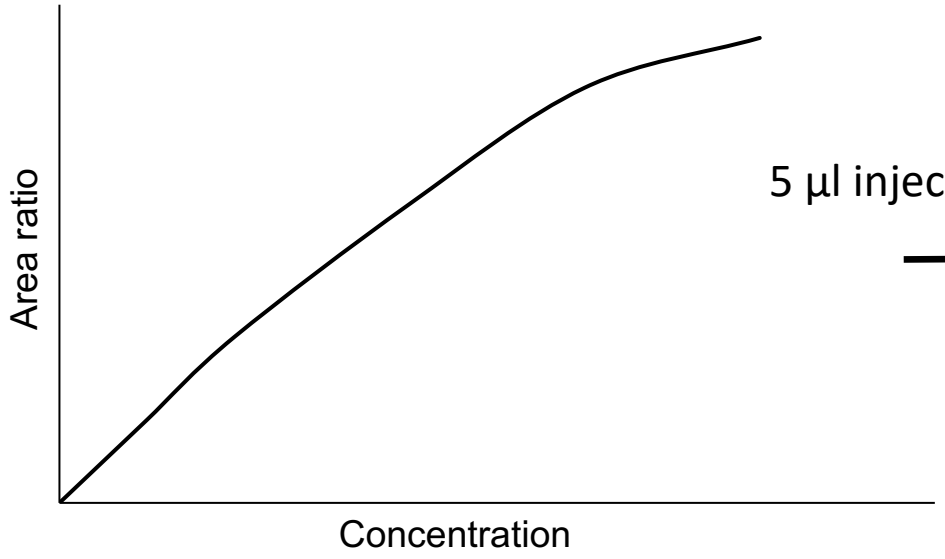
VALIDATION: CALIBRATION MODEL

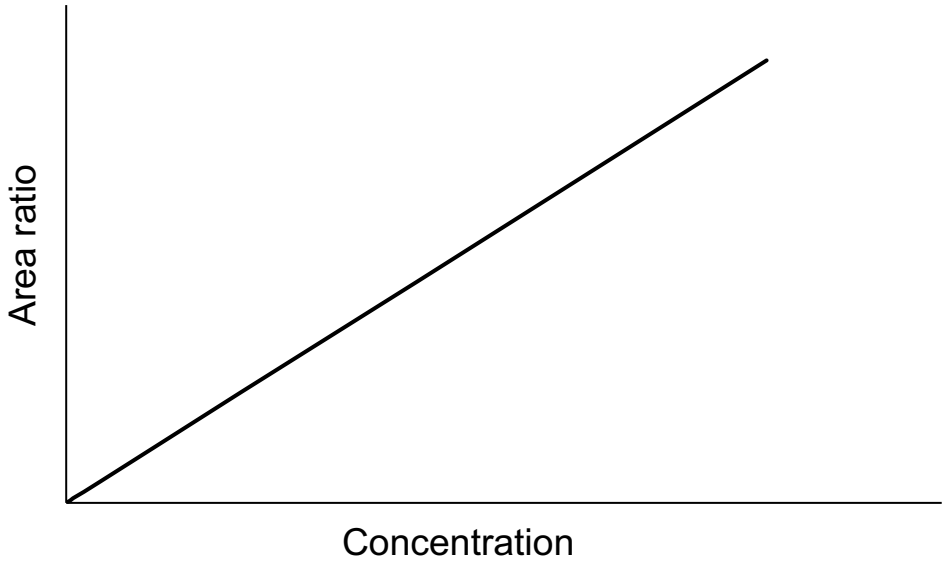
- Absence blank matrix → charcoal-treatment

- Analyte levels differed substantially between phosphatase-treated and non-treated samples

→ need for a very broad calibration range



5 µl injection of highest calibrators



Detector saturation

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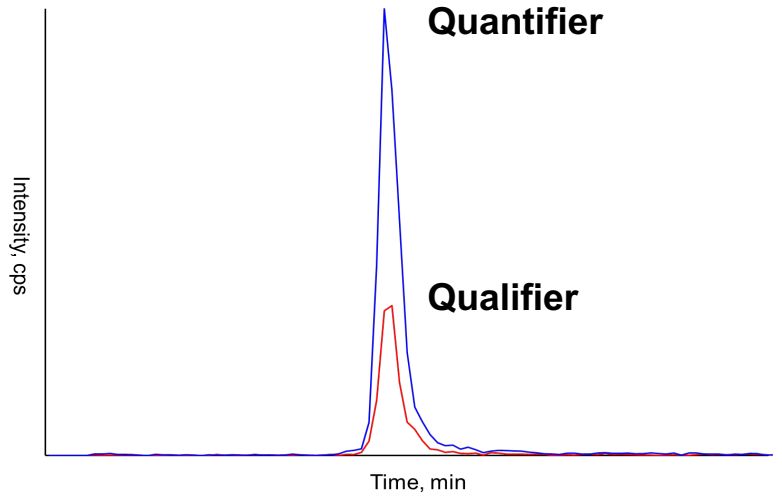
VALIDATION: SELECTIVITY

- Quantifier/Qualifier ratio
- Ion ratio's were all within the tolerated window of ion ratio neat standards solutions



		HET	HMP	Thiamine	TMP	TDP
Neats	**mean**	0.20	0.19	1.13	0.27	0.38
	tolerated window	[0.15-0.25]	[0.14-0.24]	[1.03-1.23]	[0.22-0.32]	[0.3-0.46]
	CV (%)	14%	10%	8%	12%	9%
Charcoal treated Arabidopsis	**mean**	0.20	0.19	1.14	0.26	0.37
	CV (%	9%	10%	9%	12%	7%
Spiked Arabidopsis	**mean**	0.20	0.19	1.15	0.26	0.38
	CV (%)	19%	8%	8%	10%	8%
Arabidopis	**mean**	0.25	0.18	1.14	0.26	0.37
	CV (%)	32%	9%	9%	9%	6%

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VALIDATION: MATRIX EFFECT (ME)

- Based on Matuszewski *et al.* (2003)
- Absolute ME = analyte signal matrix / analyte signal neat solvent

  → correct for endogenous signal matrix
- Relative ME= ME analyte/ ME internal standard

We demonstrated that:

- The applied internal standard compensated for matrix effect

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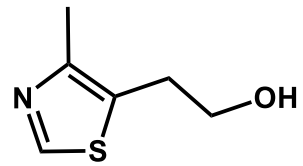
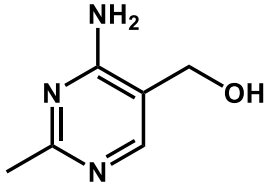
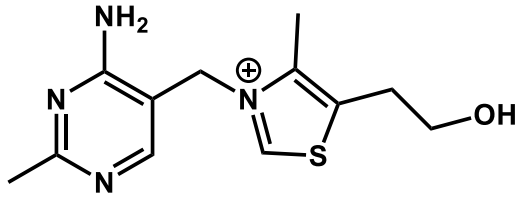
VALIDATION: MATRIX EFFECT (ME)



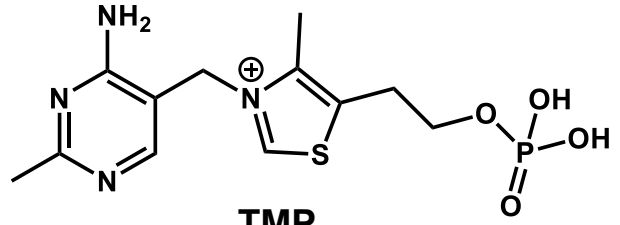
HET



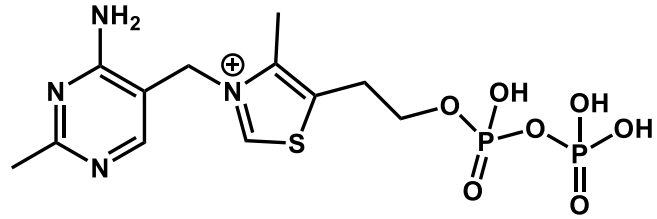
HMP



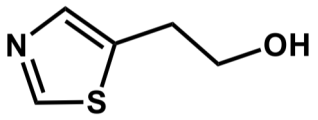
Thiamine



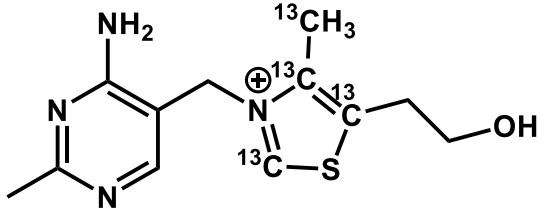
TMP



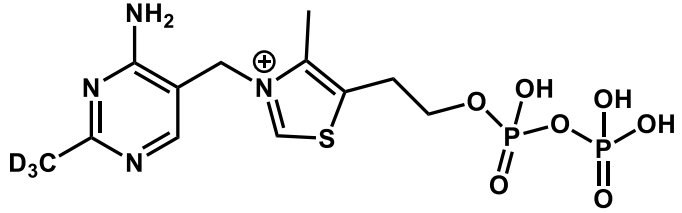
TDP



HET analogue



^{13}C Thiamine



D_3 TDP

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VALIDATION: MATRIX EFFECT (ME)

- Based on Matuszewski *et al.* (2003)
- Absolute ME = analyte signal matrix / analyte signal neat solvent

  → correct for endogenous signal matrix
- Relative ME= ME analyte/ ME internal standard

We demonstrated that:

- The applied internal standards compensated for matrix effect
- The relative matrix effect in charcoal-treated matrix was equal to the relative matrix effect in *Arabidopsis* matrix

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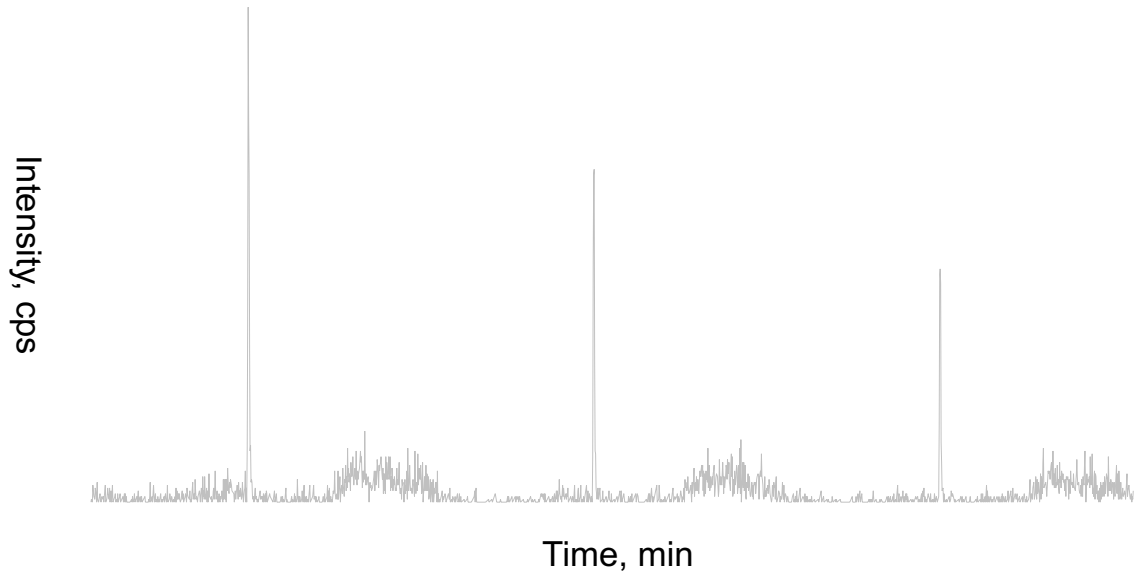
VALIDATION: CARRY-OVER

- Relevant carry-over for TMP and TDP
- Could not be improved by optimising needle wash
- Even without intervention of needle, carry-over is still an issue
- Specific measures to minimize carry-over should be taken



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VALIDATION

Parameters to evaluate:

- Selectivity ✓
- Carry-over
- Calibration model ✓
- Accuracy and precision ✓
- Dilution integrity ✓
- Matrix effect ✓
- Recovery ✓
- Stability ✓

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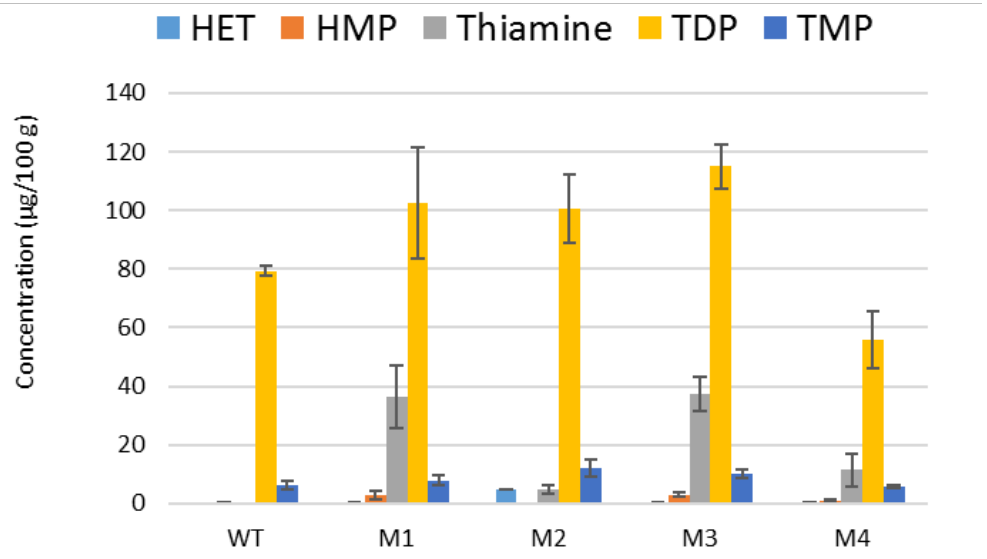
APPLICATION

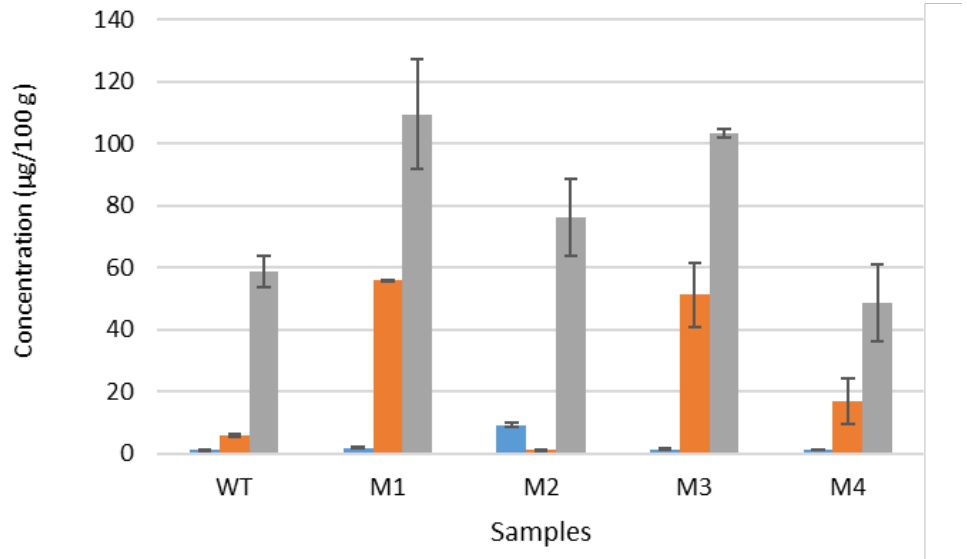
WT = non-genetically modified = ‘control’

M = genetically modified lines

Non-phosphatase treated



Phosphatase treated



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CONCLUSION

- Development of LC-MS/MS method for the quantification of thiamine, its precursors and phosphate derivatives in *Arabidopsis thaliana*
- Succesfully tackled several challenges: high polarity analytes, lability analytes, endogenous presence analytes…
- Succesful validation of the method based on international guidelines
- The method was succesfully applied on genetically modified *Arabidopsis* lines

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